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(54) Title: PEPTIDES OF NEMATODE TUBULIN AND METHODS OF USE

(57) Abstract

The present invention relates to a monoclonal antibody which substantially binds to β-tubulin of nematode origin and fragments thereof. There is provided a hybridoma cell line producing the monoclonal antibody of the present invention which has been deposited at the ATCC under the accession number HB 11129. The antibody of the present invention can be used as an anti-parasitic agent and a diagnostic agent for parasitic diseases. The present invention also relates to the use of an antigen derived from β-tubulin as an immunizing agent and in vaccine compositions.

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PEPTIDES OF NEMATODE TUBULIN AND METHODS OF USE

FIELD OF THE INVENTION

The present invention relates to a monoclonal antibody which specifically binds to β-tubulin of nematode origin, which antibody can be used as an antiparasitic agent and a diagnostic agent for parasitic diseases.

The present invention also relates to the use of immunogenic peptides useful in vaccine compositions for protecting mammals against filarial nematodes such as parasites of the *Brugia*, *Dirofilaria* and *Onchocerca* genuses.

The present invention relates to the use of peptide fragments which provide improved means to protect a mammal against parasites of the *Filarioidea*. More specifically, animals or humans exposed to the peptide fragments of the present invention are protected from infection by filarial parasites by antibodies induced by the peptide.

BACKGROUND OF THE INVENTION

Parasitic diseases such as schistosomiasis (Bilharziasis) malaria and filariasis affect large numbers of people and are frequent causes of gastrointestinal, circulatory and other disorders. Parasitic infections often are chronic or recurrent, and it is not surprising that immunologic types of diseases have been described.

Filariasis consists of a group of diseases occurring in tropical and subtropical countries caused by *Filarioidea*. The *Filarioidea* include parasites of *Brugia*, *Dirofilaria*, *Onchocerca*, *Wucheria* and *Loa* genuses. Filariasis involves the lymphatic system, with obstruction leading to chyluria, hydrocoele, and elephantiasis that may involve the scrotum, legs and the arms. Other filaria such as *Dirofilaria immitis*, infect the right heart and connecting large vessels of the canine circulatory system, causing cardiac insufficiency, pulmonary arterial disease

and right ventricular failure (canine heartworm disease). Still other filaria, such as *Onchocerca volvulus*, infect the skin and eyes of humans, causing destruction of the skin and, frequently, the retinae (river blindness).

Infection of animals and humans with filarial nematodes poses serious therapeutic problems. Prevention by prophylaxis is far superior to treatment of established infections. This is particularly true for canine heartworm disease, in which the most severe symptoms are caused by the presence of adult *Dirofilaria immitis* residing in the heart and great vessels. Because of their size and location and the low therapeutic ratios of available drugs, chemotherapeutic killing of the adult parasites can be lethal. Thus, prophylactic failure can have very serious consequences. In contrast, current chemotherapy of onchocerciasis (river blindness; due to *Onchocerca volvulus*) is primarily directed at the microfilaria. The larvae are responsible for most of the pathology in this disease. Unfortunately, adult female *O. volvulus* are quite long-lived, and repeated treatment with microfilaricides is necessary as long as the adults are viable.

The benzimidazoles are the broadest spectrum anthelmethintics available, yet they are unimpressive as microfilaricides (see Sharma et al., Adv. Drug Des., 24:200, 1993). The effects of benzimidazoles have been difficult to detect *in vitro* against many nematodes, including the filariae (Comley et al., Trop. Med. Parasitol., 39:456, 1988). Solid genetic and biochemical evidence points to nematode tubulin as the target to these drugs (see Lacey et al., Int. J. Parasitology, 18:885, 1988), and ovarian tubulin in *D. immitis* is disrupted by them (Howells and Dewells, Ann. Trop. Med. Parasitology, 79:507, 1985). It is not clear why the benzimidazoles lack clinically acceptable macrofilaricidal activity. One possibility is that these drugs have unfavorable

pharmacokinetics and fail to reach sufficient levels inside the parasites to exert the nematocidal effect. Alternatively, isotypes of filarial β-tubulins critical for viability (e.g., non-ovarian) may be inherently insensitive to these drugs. Finally, the consequences of tubulin disruption in adult filariae may not be lethal (see Geary et al., Biological Functions of Nematode Surfaces, 1994). There is reason to believe that intestinal tubulin is the major target for the benzimidazoles in gastrointestinal parasites (Bongers and DeNollin, Am. J. Vet. Res., 36:1153, 1975; Kohler and Bachmann, Mol. Biochem. Parasitology, 4:325, 1991). Similar effects on intestinal morphology have been noted in *B. malayi* after flubendazole treatment, but may have little bearing on survival of this parasite *in situ* (Geary et al., 1994, cited supra).

Etiology and Pathogenesis of Filariasis

Wuchereria bancrofti is found only in humans; *Brugia malayi* is often spread to man from animal hosts. The adult filarial worms live in the human lymphatic system. Microfilaria released by gravid females are found in the peripheral blood, usually at night. Infection is spread by many species of mosquitoes. The microfilaria are ingested by the mosquito, undergo development in the insect's thoracic muscles, and, when mature, migrate to its mouthparts. When the infected mosquito bites a new host, the microfilaria penetrate the bite puncture and eventually reach the lymphatics or bloodstream, where they develop to the adult stage. Blackflies of the genus *Simulium* are also vectors for filarial parasites, especially those in the genus *Onchocerca*. The development of the parasite in the fly and the dynamics of transmission to the host are conceptually similar to the parasite-mosquito relationship.

Pathology

Inflammation and fibrosis occurring in the vicinity of the juvenile and adult worms produce progressive lymphatic obstruction. Alternatively, the presence of adult heartworm leads to obstructive pathology in the canine heart, especially the right heart, and mechanical and/or immunologically-mediated damage to the pulmonary arteries. In river blindness, the major pathology in both the skin and eyes is mediated by the immune response of the infected human to microfilaria present in tissues.

Symptoms and signs

The incubation period may be as short as two months. The "prelatent" period, from the time of infection to the appearance of microfilariae in the blood, is at least eight months. Clinical manifestations depend on the severity of the infection; they may include lymphangitis, lymphadenitis, orchitis, funiculitis, epididymitis, lymph varices, and chyluria. Chills, fever, headache, and malaise may also be present. Elephantiasis and other late severe sequelae occur with long-time residence in endemic areas and repeated reinfection. An aberrant form of filariasis (tropical eosinophilia) is characterized by hypereosinophilia, presence of microfilariae in the tissues but not in the blood, and high titers of antifilarial antibodies (tropical eosinophilia). Clinically, the patient may present with lymphadeno-splenomegaly or with cough, bronchospasm, and chest infiltrates.

Onchocerciasis usually presents as itching and degeneration of the skin. Reduced vision is a frequent symptom of infection as well. Heartworm disease in dogs often presents as exercise intolerance, and is usually accompanied by alterations in electrocardiogram recordings or frankly altered heart sounds.

Diagnosis

Microfilariae may be found in blood, skin or lymph fluid. A number of serologic tests are available, but are not completely reliable. Antigen detection procedures are being investigated.

Tubulin

Microtubules are proteinaceous organelles that are implicated in a variety of cellular functions including mitosis, intracellular transport, the maintenance of cell shape and the formation of cilia, flagella and sensory organelles. The major structural component of microtubules is tubulin, which is composed of α- and β- subunits, the dimer having a molecular weight of 110 kg. Both α- and β-tubulins are expressed as heterogeneous but closely related families of multiple isoforms, in different organisms, tissues and even within single cells of the same organism. The heterogeneous population of tubulin isoforms may result from both the differential expression of distinct tubulin genes and post-translational modifications. It has been suggested that the diversity in tubulin isoforms may have implications for specific MT functions (Lewis and Cowan, J. of Cell Biol., 106:2023-2033, 1988). The precise nature or role of α- and β-tubulin isoforms have not yet been elucidated, although several groups of researchers have demonstrated that many *in vivo* functions of tubulin are to some extent, isoform specific (Gundersen et al., Cell, 38:779-789, 1984).

Benzimidazoles, anti-mitotic and anti-fungal agents are widely used in the chemotherapy of parasitic diseases. Several chemicals such as colchicine, vinblastine and benzimidazoles have been shown to bind to tubulin. Benzimidazoles exert toxic effects on nematodes *in vitro* by binding to tubulin and inhibiting polymerization of the heterodime into microtubules.

Benzimidazoles induce paralysis and slow growth in the free-living nematode *Caenorhabditis elegans*. However, the precise benzimidazoles binding site has not been determined.

Monoclonal antibodies have made it possible to recognize different domains of tubulin in different species in order to study the structure, distribution and functions of tubulin. Tang and Prichard (Mol. & Biochem. Parasitology, 32:145-152, 1989) reported the presence of 4 to 5 β-tubulin isoforms in the tubulin-enriched extracts of adult *B. pahangi*. In addition, immunogold studies with *B. malayi* adult and microfilariae using anti-tubulin monoclonal antibodies have revealed the presence of β-tubulin in the somatic muscle blocks beneath the cuticle, intestinal brush border and intra-uterine microfilariae of the adult worms (Helm et al., Parasite Immunology, 11:479-502, 1989).

Several other anti-tubulin monoclonal antibodies raised against parasitic protozoa and nematodes have been isolated but these have been found to cross-react with tubulin from other species. For example, Draber et al. (Protoplasma, 128:201-207, 1985) reported a monoclonal antibody raised against pig brain tubulin which reacted with microtubules from diverse species (mammalian, bird, amphibian, fungi, echinoderm, platyhelminth, slime moulds) but not protozoan tubulin. Similarly, Birkett et al. (FEBS Letters, 187:211-218, 1985) generated an anti-β-tubulin monoclonal antibody against *Physarum myxamoebae* which reacts with β-tubulin from various fungi, algae, higher plants, avian, insect and several mammalian sources. In addition, Helm et al. (Parasite Immunology, 11:479-502, 1989) have raised monoclonal antibodies against microfilariae of *Brugia* species. Contrary to the anti-*B. pahangi* β-tubulin monoclonal antibodies of the present

invention, their monoclonal antibodies cross-reacted with mammalian tubulin.

All these monoclonal antibodies of the prior art are not specific against tubulin of nematode origin.

It would be highly desirable to have a monoclonal antibody which specifically binds to nematode tubulin and which could be used as an anti-parasitic agent and as a reliable diagnostic agent for parasitic diseases.

It would be also highly desirable to have a peptide which can be used to immunize mammals against parasites such as *Brugia*, *Dirofilaria*, and *Onchocerca*.

The desired peptide could be used in vaccine composition to provide an immune protection against these parasites.

SUMMARY OF THE INVENTION

In accordance with the present invention there is provided a monoclonal antibody which specifically binds to β-tubulin of nematode origin and fragments thereof. The monoclonal antibody of the present invention can be used as an anti-parasitic agent and as a diagnostic agent for parasitic diseases.

In accordance with the present invention, there is also provided a hybridoma cell line which produces the monoclonal antibody of the present invention.

The monoclonal antibody of the present invention recognizes the C-terminal of nematode β-tubulin which corresponds to a peptide of eighteen amino acids.

In accordance with another embodiment of the present invention, there is provided the use of a peptide as an immunizing agent against parasites wherein said immunizing agent comprises at least one peptide that has a sequence that corresponds to the C-terminal amino acids of B-tubulin from a filarial parasite. An example of such a peptide corresponding to the sequence of the C-terminal

peptide of *B. pahangi* has the following amino acid sequence:

Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu
1 5 10 15

Gln Glu Glu.

The use of the peptide from *B.pahangi* in accordance with the present invention induces by a host the production of cytotoxic antibodies against parasites such as *Brugia* and *Dirofilaria*.

In accordance with the present invention, there is also provided a vaccine for parasite infection comprising at least one peptide having an amino acid sequence of the carboxy terminal end of B-tubulin from a filarial parasite. An example of such a peptide is that which corresponds to the C-terminal amino acids of *B. pahangi* and which has the following amino acid sequence:

Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu
1 5 10 15

Gln Glu Glu.

Another example of such a peptide is a peptide that has a sequence that corresponds to the C terminal amino acids of *Dirofiliria immitis*. A peptide derived from *Dirofilaria* immitis can have a sequence:
Asp Glu Asp Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln Glu Glu.

Another example of such a peptide is a peptide that has a sequence that corresponds to the C terminal amino acids of *Oncocerca volvolus*. A peptide derived from *Onchocerca volvolus* can have an amino acid sequence:
Asp Asp Glu Ala Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln Glu Glu.

Finally, in accordance with the present invention, there is provided a method of immunizing mammals against parasites comprising the administration of the

vaccine of the present invention. The vaccine of the present invention can be administered in a dosage range of 0.015 μg to 0.15 mg per kg body weight, preferably in a dosage range of 1.5 μg to 0.15 mg per kg body weight.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the nature of the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and wherein:

Fig. 1 is a Western blot analysis of anti-*B. pahangi* and anti-chick brain tubulin monoclonal antibody to different proteins;

Fig. 2 is a Western blot analysis of anti-*B. pahangi* tubulin monoclonal antibodies to the total protein extract of adult *B. pahangi*;

Fig. 3 is the peptide mapping and Western blots of *B. pahangi* tubulin;

Fig. 4 is a graph of the effects of anti-*B. pahangi* tubulin monoclonal antibody P3D on the viability of adult female *B. pahangi in vitro*;

Fig. 5 is a graph of the effects of anti-*B. pahangi* tubulin monoclonal antibody 1B6 on the viability of adult female *B. pahangi in vitro*; and

Fig. 6 is a graph of the effects of anti-chick brain tubulin monoclonal antibody 357 on the viability of adult female *B. pahangi in vitro*.

Fig. 7 shows the cDNA sequence encoding β-tubulin from *B. pahangi*, *Dirofilaria immitis*, and *Onchocerca volvulus*.

Fig. 8 shows the predicted amino acid sequence of β-tubulins from *B. pahangi*, *D. immitis*, and *Onchocerca volvulus*.

DETAILED DESCRIPTION OF THE INVENTION

I-Monoclonal antibody

A first embodiment of the present invention relates to the production and characterization of a monoclonal anti-*B. pahangi* tubulin monoclonal antibody.

The monoclonal antibody of the present invention, denoted P3D, specifically reacts to the C-terminal portion of β-tubulin from *B. pahangi* and *Dirofilaria* and hence is capable of killing these parasites. The hybridoma P3D producing the monoclonal antibody of the present invention has been deposited at the American Type Culture Collection (12301 Parklawn Drive, Rockville, Maryland, USA 20852) under accession number HB 11129 on September 18, 1992. This deposit is available to be public upon the grant of a patent to the assignee, McGill University, disclosing same. The deposit is also available as required by Foreign Patent laws in countries wherein counterpart applications are being filed.

In total, fifty-four anti-*B. pahangi* tubulin monoclonal antibodies were obtained after immunization of mice with purified *B. pahangi* tubulin. Because of their remarkable specificity for tubulin, monoclonal antibodies P3D and 1B6 among others, have been selected for more extensive characterization. Western blot analysis of one-dimensional SDS-PAGE showed that the anti-*B. pahangi* monoclonal antibodies of the present invention recognized tubulin from a number of filarial nematodes (*B. pahangi*, *B. malayi* and *D. immitis*) and an intestinal nematode (*H. contortus*). However, the monoclonal antibodies did not cross-react with tubulin from pig brain, 3T3 mouse fibroblast cells or the parasitic protozoan *G. muris*. On the other hand, anti-chick monoclonal antibody 357 reacted with pig brain, 3T3 mouse fibroblast and *G. muris* tubulins as strongly as it did with filarial and other nematode β-tubulins. The anti-*B. pahangi* tubulin monoclonal

antibodies of the present invention recognize an epitope that is conserved between filarial and intestinal nematode β-tubulin but not in protozoan and mammalian β-tubulin. The epitope recognized by monoclonal antibody 357 has been localized to a region of β-tubulin between amino acid 339-417 in the proteolytic fragments of pig brain tubulin (Serrano et al., Analytical Biochemistry, 159:253-259, 1986). The anti-*B. pahangi* tubulin monoclonal antibodies of the present invention are highly specific to nematode tubulin.

The monoclonal antibodies of the present invention specific for the α- or β-subunit of tubulin allow the subcellular localization and the function of each subunit of tubulin to be studied. Proteins of the size of tubulin are generally built of several structural domains that have distinct functions. In the case of tubulin, such functions include binding of anti-microtubule drugs, GTP or microtubule-associated proteins and the association between monomers, dimers or protofilaments. The nematode-specific anti-tubulin monoclonal antibodies of the present invention may serve to characterize the structure and distribution of *B. pahangi* tubulin molecule, and to define microtubule stability and functional domains.

The following procedures are used in the preparation and the characterization of the monoclonal antibody of the present invention.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed in microtiter plates (Falcon) coated with the polylysine-purified tubulin or an 18 amino acid peptide corresponding to the extreme C-terminal residues 430-448 of *B. pahangi* tubulin (Guénette et al., Mol. & Biochem. Parasitology, 44:153-164, 1991) at a concentration of 10μg/ml in phosphate buffer saline (PBS). Plates are incubated with 200 μl of 1% bovine serum albumin

(BSA) in PBS. Horseradish peroxidase-labeled anti-mouse IgG or IgM (Bio-Can, Mississauga, Ontario) at dilutions of 1:5000 and 1:20,000, respectively, is added to each well and incubated for 1 hour at 37°C. The substrate is 2,2'-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) (Sigma). The plates are read on a Titertek multiskan™ plate (Flow Laboratories, Irvine, Ayrshire, UK) at 414 nm. Normal mouse serum or culture medium used to grow hybridoma cells (Iscoves modified Dulbecco's medium (IMDM) with 20% FCS, 10% NCTC 135 and HT) is used as a negative control.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples are run in a Mini Protean II™ dual slab cell (Bio-Rad, Richmond, CA) using 4% polyacrylamide as stacking and 12% polyacrylamide as separating gels.

Isoelectric focusing and two-dimensional electrophoresis (IEF-2D SDS-PAGE)

IEF gels are prepared and run in tube gels (1.5 x 8 cms) containing 9.5 M urea (LKB) and 2% (w/v) ampholines (LKB) (1.6% pH 4-6 and 0.4% pH 3.5-10). IEF is conducted at 400 V for a period of 16 hours and then at 800 V for 3 h. Electrophoresis is performed in 4% polyacrylamide stacking and 12% polyacrylamide separating gels, running at 50 V for 30 min and at 150 V for 60 min, in the Mini Protean II™ slab cell. After 2-dimensional (2D) SDS-PAGE, gels are either stained with silver stain (Bio-Rad), or the proteins are transferred onto nitrocellulose (NC) sheets for Western blot analysis.

Western blotting

After 1 and 2D SDS-PAGE, tubulin subunits, individual tubulin isoforms and peptides are electrophoretically transferred onto nitrocellulose sheets

for 2 hours at 4°C. The nitrocellulose sheets are cut into several strips containing an identical pattern of separated proteins. To visualize protein bands, two nitrocellulose strips are stained with amido black. The remaining strips are washed in PBS and incubated for 2 hours at room temperature in 10% newborn calf serum (Gibco) in Tris-buffer saline (140 mM $NaCl_2$, 50 mM Tris-HCl, pH 7.4, with 0.1% (v/v) Tween 20™ (TBS-T)) to saturate the unoccupied protein binding sites of the nitrocellulose. After washing, the strips are incubated overnight at 4°C with anti-tubulin monoclonal antibodies (MAbs) or IMDM (negative control). The nitrocellulose strips are then washed 6 x 5 min with TBS-T, immersed in peroxidase-conjugated goat anti-mouse IgM or IgG (Bio-Can) diluted at 1:500 with high salt buffer (1 M $NaCl_2$, 10 mM Tris-HCl, pH 7.4; 0.5% (v/v) Tween 20™ (HSB-T) with 10% NBCS), and incubated for 2 hours at room temperature. After washing the nitrocellulose strips with TBS-T for 30 min, the bound peroxidase is detected with the substrate 4-chloro-1-naphthol (Sigma) at 3 mg/ml in methanol/PBS, 1:5 (vol/vol), containing 0.075% of 30% hydrogen peroxide.

1. <u>Preparation of antigens</u>

Gerbils (<u>Meriones unguiculatus</u>), 9-10 months old and previously infected intraperitoneally with 400 *B. pahangi* infective larvae, are obtained from Dr. J. McCall (University of Georgia, USA). The adult *B. pahangi* (0.7 g) are harvested from the peritoneal cavities of gerbils in warm physiological saline (0.85% NaCl), washed with 0.025 M buffer containing 1 mM ethyleneglycol-bis-(β-aminoethylether)N,N,N',N'-tetraacetic acid (EGTA), 0.5 mM $MgSO_4$ and 1 mM guanosine-5'-triphosphate (GTP), and are homogenized in 7 ml of 2[N-morpholino]-ethanesulfonic acid (MES) buffer. The homogenate is centrifuged at 100,000 g for 1 hour at 4°C. The supernatant is retained and the

pellet discarded. The same procedure is used to prepare tubulin from other filarial (*B. malayi* and *D. immitis*) and non-filarial nematodes (*A. suum*, benzimidazole-susceptible and resistant strains of *H. contortus*).

Tubulin from pig brain is prepared by 2 cycles of polymerization-depolymerization.

Giardia Muris antigen is prepared as a sonicate.

A peptide corresponding to amino acid residues 430-448 of *B. pahangi* β-tubulin, synthesized using an Applied Biosystems Peptide Synthesizer™, HPLC purified, sequenced and coupled to the carrier protein, keyhole Limpet Hemocyanin (KLH) (The Alberta Peptide Institute), is also used as an antigen in enzyme-linked immunosorbent assay (ELISA).

2. **Purification of parasite tubulin**

B. pahangi, *B. malayi*, *D. immitis*, *A. suum* and *H. contortus* tubulins are partially purified using polylysine affinity chromatography (Lacey & Prichard, Mol. & Biochem. Parasitology, 19: 171-181, 1986). The elution profile consisted of three distinct peaks. The first protein peak is eluted with MES buffer, the second with 1% aqueous $(NH_4)_2SO_4$. Fractions for each peak are pooled and concentrated separately in centriflo™ (Amicon) at 400 g.

Polylysine-purified proteins are separated on SDS-PAGE, protein bands of the molecular weight corresponding to tubulin are excised, and the protein is electro-eluted (Electroeluter™, Bio-Rad) (Blose et al., J. of Cell Biol., 98: 847-858, 1984). The eluted protein is precipitated three times with 80% acetone at -20°C for 5 hours and then dissolved in 0.125 M Tris-HCl (pH 6.8), 0.1% SDS and 1 mM EDTA, dialysed overnight against this buffer at 4°C and stored at -70°C until used.

Crude supernatant of adult *B. pahangi* is chromatographed on a polylysine agarose column. The

protein content of each fraction is determined. The elution profile consisted of 3 distinct protein peaks. The protein concentrations in the first and second peaks are very high compared with that in the third peak, but in contrast to this last peak the first two peaks contains little if any tubulin. This is consistent with the previous report by Tang & Prichard (Mol. & Biochem. Parasitology, 32: 145-152, 1989). Third peak proteins are concentrated and then subjected to SDS-PAGE, respectively. The tubulin band is cut out of the SDS-gels and subjected to electro-elution for further purification.

3. Immunization and preparation of monoclonal antibodies

Six week old female BALB/c mice (Charles River Canada Inc., St. Constant, Québec) are injected subcutaneously at three week intervals with purified eluted *B. pahangi* tubulin (100 µg/injection) using equal volumes of complete Freund's adjuvant for the first injection and incomplete adjuvant for the second injection. The third immunization of 100 µg of tubulin in PBS is administered intraperitoneally (i.p.). At this stage, mice are bled and serum is tested for anti-tubulin antibodies by ELISA and Western blotting. The spleen cells from the mouse giving the highest titer are fused with the myeloma cell line, P3X63.Ag8 (American Type Culture Collection (ATCC), accession number CRL1580, Rockville, MD), as described by Hurrell ("Monoclonal hybridoma antibodies: Techniques and applications", 1983, CRC Press, Boca Raton, Florida, p. 22). Positive cultures as determined by ELISA and Western blotting, are cloned twice by limiting dilution.

Two different isotypes of anti-*B. pahangi* monoclonal antibodies were obtained. Seven out of fifty-four monoclonal antibodies were polyreactive IgM, recognizing tubulin as well as other high and low molecular weight proteins, whereas the remaining monoclonal

antibodies represented two populations of the IgG isotype. Of these, four out of fifty-four reacted with tubulin and other low molecular weight proteins; however, forty-three monoclonal antibodies were specific for tubulin. Monoclonal antibodies P3D and 1B6 specific to nematode tubulin, were chosen for further characterization. These monoclonal antibodies are of IgG isotype.

4. Monoclonal antibodies (MAbs)

Three monoclonal antibodies, all specific for tubulin, are investigated. Anti-chick brain monoclonal antibody 357, which cross-reacts with β-tubulins from a spectrum of eukaryotic cell types, was purchased from the Radiochemical Centre (Amersham, England) and monoclonal antibodies P3D and 1B6 are raised against the tubulin of adult *B. pahangi*. All anti-tubulin monoclonal antibodies are of IgG isotype.

5. Specificity of monoclonal antibodies (MAbs) P3D, 1B6 and 357

The specificity of these monoclonal antibodies is investigated by determining their reactivity to proteins from a variety of filarial and non-filarial nematodes, protozoa and mammalian cells using ELISA and Western blot.

In ELISA, the anti-*B. pahangi* monoclonal antibodies P3D and 1B6 do not react with *G. muris* tubulin, which is recognized by anti-chick brain tubulin monoclonal antibody 357. Crude and partially purified extracts of adults and microfilariae of *B. pahangi*, adult *B. malayi* and *D. immitis*, egg of *H. contortus*, adult *A. suum*, pig brain and 3T3 mouse fibroblast cell tubulins are separated on SDS-PAGE and electrophoretically transferred onto nitrocellulose sheets. The blots are treated with: (1) amido black; (2) monoclonal antibody 1B6; (3) monoclonal antibody P3D; and monoclonal antibody 357.

Analysis of amido black stained blots revealed that crude extracts of adults and microfilariae of *B. pahangi*, adult *B. malayi* and *D. immitis*, eggs of susceptible and resistant strains of *H. contortus*, adult *A. suum*, pig brain and 3T3 mouse fibroblast cell contained many bands in the tubulin region. Tubulin from the various nematodes and mammalian extracts are separated into two bands designated α and β. Anti-*B. pahangi* monoclonal antibody P3D recognized specifically β-tubulin from adult and microfilariae of the filarial worms *B. pahangi*, *B. malayi* and *D. immitis* (Fig. 1A, lane 1-4). It also reacted with equal intensity to tubulin from the intestinal nematode *H. contortus* (BZ-susceptible and benzimidazole-resistant strains) (Fig. 1A, lane 5-6). Tubulin from *A. suum* do not show very strong reactivity with this monoclonal antibody (Fig. 1A, lane 7), no reactivity to 3T3 mouse fibroblast cells or pig brain tubulins is detected (Fig. 1A, lane 8-9). Similar results are obtained using monoclonal antibody 1B6 (not shown). Whereas, cross-reactive anti-chick β-tubulin monoclonal antibody 357 recognized β-tubulin from all nematodes and mammalian cells (Fig. 1B, lane 1 to 9).

6. <u>Identification of tubulin isoforms</u>

Anti-*B. pahangi* β-tubulin monoclonal antibodies P3D and 1B6, and anti-chick β-tubulin monoclonal antibody 357, are used to characterize β-tubulin isoforms in *B. pahangi* tubulin. Monoclonal antibodies P3D (Fig. 2A) and 357 recognized the same isoform pattern, reacting with two β-tubulin isoforms in the crude as well as partially purified extracts of *B. pahangi* (not shown). Whereas, monoclonal antibody 1B6 specifically recognized only one β-tubulin isoform in the extract of *B. pahangi* (Fig. 2B). The β-tubulin isoforms are in the pH range of 5.1-5.3.

Monoclonal antibody 357 probed blots are re-probed with monoclonal antibodies P3D and 1B6 respectively,

to demonstrate that the same spots are recognized by this monoclonal antibody. Furthermore, to show the full complement of β-tubulin isoforms, P3D and 1B6 probed blots are re-probed with monoclonal antibody 357. The results indicated that all these monoclonal antibodies recognized the same isoforms in tubulin-enriched extracts of adult *B. pahangi*. However, monoclonal antibody 1B6 is specific to one isoform.

7. Limited proteolysis of tubulin

Limited proteolysis of tubulin in gel slices is performed. Gel pieces corresponding to the tubulin are cut out of the polyacrylamide gels and placed directly into the sample well of a second 15% SDS-polyacrylamide gel. Gel pieces are overlaid with one of the following proteases: α-chymotrypsin from bovine pancreas (Sigma) or *S. aureus* V8 protease (Boehringer Mannheim). The SDS-PAGE is performed at 50 V until bromophenol blue due reached the bottom of the stacking gel and then increased to 150 V for the remainder of the electrophoresis. After SDS-PAGE, the digested peptides are either stained with silver stain or transferred onto nitrocellulose sheets, in the same way as described for the Western blot analysis, and reacted either with anti-*B. pahangi* tubulin monoclonal antibodies or anti-chick tubulin monoclonal antibody 357.

8. Interaction of anti-tubulin monoclonal antibodies with tubulin proteolytic fragments

Three identical gels are run and the peptide fragments transferred onto nitrocellulose, three of which are immunostained with anti-*B. pahangi* tubulin monoclonal antibodies P3D and 1B6 (Fig. 3) and anti-chick brain β-tubulin monoclonal antibody 357 (not shown).

Western blots of peptides digested with chymotrypsin showed that monoclonal antibody P3D reacted

with a 21 kDa chymotrypsin fragment (Fig. 3A, lane 2) and a 21 kDa V9 protease β-tubulin fragment (Fig. 3A, lane 3). In contrast, monoclonal antibody 1B6 reacted with the two chymotrypsin-digested fragments of 42 and 34 kDa (Fig. 3B, lane 2). It reacted strongly with the 42 kDa and weakly with the 34 kDa fragment. However, the same protease (Fig. 3B, lane 3). These results of the limited proteolysis analysis indicate that the antigenic site recognized by monoclonal antibody P3D differs from that recognized by monoclonal antibody 1B6.

Although monoclonal antibody 357 reacts strongly to intact β-tubulin from *B. pahangi*, no interaction was seen with β-tubulin fragments digested with chymotrypsin or V8 protease (not shown). Protease digestion appears to destroy the reactivity of *B. pahangi* tubulin towards monoclonal antibody 357.

II-Peptide used as an immunizing agent against parasite

The monoclonal antibody P3D of the present invention recognizes the C-terminal of nematode β-tubulin which corresponds to a peptide of eighteen amino acids.

A second embodiment of the present invention relates to the use of a peptide derived from the C-terminus of nematode B-tubulin. The antibody of the present invention recognizes a peptide which includes the following eighteen amino acid sequence:

Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu
1 5 10 15

Gln Glu Glu,

which is located at the C-terminal of nematode β-tubulin of *B.pahangi*.

Furthermore, the present invention relates to a vaccine which comprises at least one peptide that has an amino acid sequence that corresponds to the amino acids at

the C terminus of B-tubulin. A specific example of such a peptide has the following amino acid sequence:

Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu
1 5 10 15
Gln Glu Glu.

The present invention provides a peptide having the amino acid sequence derived from the eighteen amino acids at the C-terminal of β-tubulin from such parasites as *Brugia*, *Dirofilaria*, and *Onchocerca*. The peptide can be made using a peptide sequence or using recombinant DNA technology.

A vaccine comprising the peptide of the present invention, a fragment thereof or a larger peptide which comprises the amino acid sequence of the peptide of the present invention is effective in conferring protection against parasite infection. Such vaccines can be prepared by one having ordinary skill in the art.

It has been discovered that monoclonal antibodies which specifically react to the C-terminal portion of β-tubulin from *Brugia* is capable of killing these parasites.

Accordingly, using a vaccine that comprises peptide with the epitope of the C-terminal of *B. pahangi* β-tubulin will elicit cytotoxic antibodies in vaccinated mammals that can kill these parasites and therefore protect the mammal against the parasite.

The present invention relates to vaccines which comprise a peptide having the sequence of about eighteen amino acid residues from the C terminus of closely related filarial parasites such as *Brugia*, *Dirofilaria* or *Onchocerca* β-tubulin or fragment thereof and to vaccines which comprise a peptide that have portions which are the eighteen amino acid sequence.

The amino acids at the carboxy terminus of *Brugia* β-tubulin are:

DEEGDLQEGESEYIEQEE or

Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu
1 5 10 15

Gln Glu Glu,

or aspartate-glutamate-glutamate-glycine-aspartate-leucine-glutamine-glutamate-glycine-glutamate-serine-glutamate-tyrosine-isoleucine-glutamate-glutamine-glutamate-glutamate.

The amino acids at the carboxy terminus of *Dirofilaria* β-tubulin are:
DEDGDLQEGESEYIEQEE or Asp Glu Asp Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln Glu Glu.

The amino acids at the carboxy terminus of *Onchocerca* β-tubulin are:
DDEADLQEGESEYIEQEE or Asp Asp Glu Ala Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln Glu Glu.

The carboxy terminus amino acids of β-tubulin from the filarial parasites are highly conserved among different filarial parasites. Peptide vaccines can be prepared therefore including one or more peptides derived from the C-terminus of β-tubulin. It is preferred that a vaccine composition for a particular parasite includes the peptide derived from that parasite.

The size of the peptide is preferably about 18 amino acids. However, the size of the peptide can be larger as long as the 18 amino acids are included and are antigenic. When prepared by automated synthesis, preferably the peptide is no larger than 50 amino acids. Smaller portions of the peptides can also be generated with a minimum size of about 4 to 7 amino acids. Smaller portions or fragments of the peptide are preferably attached to a larger carrier agent.

The sequence of each of the peptides can be preferably modified by conservative amino acid substitutions at one or more locations but preferably at one or two amino acid residues. Conservative amino acid

substitutions are known to those of skill in the art and are described in Dayhoff Atlas of Protein Sequence and Structure 5 (1978) and Argos in EMBO J. 8:779 (1989). Amino acid substitutions at position 2, 3 or 4 of an 18 amino acid peptide are especially preferred. For example, the sequence of the peptide derived from *Dirofilaria immitis* when compared with that of *B. pahangi* has a single amino acid substitution at the third amino acid residue. In the *Dirofilaria* peptide, an aspartic acid is substituted for glutamic acid at amino acid residue 3. Another example is the sequence of the peptide derived from *Onchocerca volvulus*. The sequence of the peptide from *O. volvulus* has a substitution at amino acid residues 2 and 4 when compared with the sequence for *B. pahangi*. The *Onchocerca volvulus* sequence has an aspartic acid instead of a glutamic acid at residue 2 and an alanine instead of a glycine at residue 4.

In preparing the peptide for a vaccine composition, it especially preferred that the peptide is attached to a larger carrier agent. Examples of carrier agents include bovine serum albumin, keyhole limpet hemocyanin, MAP (multiple antigen peptide) and the like. Methods of attaching peptides to carrier molecules are known to those of skill in the art. The preferred carrier molecule is MAP available from NovaBiochem (Switzerland). The peptide is synthesized on MAP following the method of Tam et al., J. Biol. Chem., 263:1719 (1988); PNAS, 85:5409 (1988).

A vaccine composition can also include a pharmaceutical acceptable diluent or excipient. Pharmaceutically acceptable diluents or excipients are known to those of skill in the art and include physiological saline, Ringer's solution and the like. Optionally, the vaccine composition may include an adjuvant. Adjuvants include incomplete Fruend's adjuvant and the like.

The vaccine composition can be administered through a variety of routes. The routes of administration include parenterally, intramuscularly, subcutaneously intraperitoneally, intravenously, and orally. The preferred route of administration is intramuscular.

A vaccine composition is administered in a single dose or multiple doses as is necessary to provide protection against the filarial infection. Protection against filarial infection can be measured by a decrease in worm burden or a decrease in filarial load or both.

In a preferred version for protection against *Dirofilaria immitis* a peptide having the following sequence: DEDGDLQEGESEYIEQEE is coupled to the carrier agent MAP using the method of Tam et al., cited supra. The peptide-MAP conjugate is combined with incomplete Freunds adjuvant. About 100 ug/dog of the peptide MAP conjugate is injected into dogs at 2 weeks, 4 weeks and 6 weeks before infection. Dogs are challenged with 40 adult *Dirofilaria immitis* subcutaneously 2 weeks after last vaccination. A change in the course of *Dirofilaria* infection can be monitored by measuring microfilaria population in blood, and population of adults in the circulatory system at autopsy.

Production of the peptide of the present invention, fragment thereof or larger peptides which include this sequence can be accomplished using standard peptide synthesis or recombinant DNA techniques both well known to those having ordinary skill in the art. Peptide synthesis is the preferred method of making polypeptides which comprise about 50 amino acids or less. For larger molecules, production in host cells using recombinant DNA technology is preferred.

Smaller peptides according to the present invention can be synthesized, for example, by solid-phase methodology utilizing an Applied Biosystems 430A peptide

synthesizer (Applied Biosystems, Foster City, California) as described in detail below.

For larger molecules, production in host cells using recombinant DNA is preferred. There are several different methods available to one having ordinary skill in the art who wishes to use recombinant DNA technology to produce proteins. Typically, genes encoding desired polypeptides are inserted in expression vectors which are then used to transform or transfect suitable host cells. The inserted gene is then expressed in the host cell and the desired polypeptide is produced.

Methods and materials for preparing genes and recombinant vectors, transforming or transfecting host cells using the same, replicating the vectors in host cells and expressing biologically active foreign peptides and proteins are described in Principles of Gene Manipulation, by Old and Primrose, 2nd edition, 1981 and Sambrook et al., Molecular Cloning, 2nd Edition, Cold Spring Harbor Laboratory Press, NY (1989), the disclosure of both is incorporated herein by reference.

European Patent application 322,237 published on June 28, 1989, U.S. Patent 4,735,801 (Stocker, April 5, 1988) and U.S. Patent 4,837,151 (Stocker, June 6, 1989) describe attenuated microorganisms useful in vaccine and which microorganisms have non-reverting mutation in two discrete genes in their aromatic biosynthetic pathway. These microorganisms can usefully form the basis of an oral live vaccine and can be genetically engineered so as to express antigens from other pathogens. These references are all incorporated herein by reference.

EXAMPLE I

In vitro assay of B. pahangi inhibition

Measurement of the *in vitro* activity of anti-*B. pahangi* tubulin monoclonal antibodies P3D, 1B6 and anti-

chick brain tubulin monoclonal antibody 357 against female *B. pahangi* or anti-chick tubulin monoclonal antibodies can independently cause any damage to the intact adult worms. Mebendazole (MBZ) is used to determine whether the presence of MBZ drug alone or in synergy with monoclonal antibodies has any differential effect.

Inhibitors

Anti-*B. pahangi* β-tubulin monoclonal antibodies P3D, 1B6 (in culture medium), anti-chick β-tubulin monoclonal antibody 357 (in ascites fluid) and mebendazole (MBZ) (in DMSO), a benzimidazole anthelmintic drug, are used as inhibitors in the *in vitro* assays. Anti-*B. pahangi* anti-chick brain monoclonal antibody 357 is in ascites fluid and is diluted to 1:1000 concentration with culture medium IMDM/FCS.

Culture *in vitro*

Parasitic nematodes are isolated from their mammalian host. *B. pahangi* are isolated from peritoneal cavities of gerbils, as described earlier in a sterile hood of Iscove's Modified Dulbecco's Medium/NCTC-135 supplemented with 20% fetal calf serum (IMDM/FCS). Following isolation, *B. pahangi* are washed five times with sterile IMDM/FCS medium, for surface sterilization. Three wells in 24-well plates (Nunc) are set up for each test monoclonal antibody, drug and for the control cultures. To each well was added 2 ml of the appropriate test medium containing pure monoclonal antibody P3D, 1B6, 357 alone or monoclonal antibody and MBZ and two adult worms. The plates are incubated at 37°C in a humidified incubator in the presence of 95% air and 5% CO_2. Worm activity is observed every two hours, and motility is assessed subjectively by observation with a naked eye. Experiment is terminated after 48 hours. During the 48 hour

incubation the culture medium is not changed. Control medium contained an identical volume of the IMDM/FCS without monoclonal antibodies or drug.

Optimization of MTT reduction assay

Female live *B. pahangi* worm is place in 0.5 ml of IMDM containing 0.5 mg/ml [3-(4,5-dimethyl(thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma) (MTT) and incubated at 37°C for various time intervals ranging from 0-90 min (MTT-reduction). Female worms that had previously been heat-killed are also incubated with MTT for selected time intervals over this range. For each time point three replicate worms are used. At the end of the MTT incubations worms are removed and carefully transferred to a separate well of a microtiter plate containing 200 µl of DMSO and allowed to stand at room temperature for 1 hour (formazan solubilization), with occasional gentle agitation to evenly disperse the color. The absorbance of the resulting formazan solution is then determined at 550 nm, using an ELISA reader and compared with a DMSO blank.

Quantification of *B. pahangi* viability

A three-step colorimetric assay based on MTT is used to assess viability of parasitic nematodes. MTT is dissolved in PBS at a concentration of 5.0 mg/ml and subsequently diluted to 0.5 mg/ml with PBS. Worms are incubated for 30 min at 37°C (MTT reduction). After incubation, worms are transferred to 96 well plates containing 200 µl of DMSO. The plates are allowed to stand for 1 hour at room temperature (formazan solubilization). The absorbance is determined at 550 nm in the presence and absence of worm and compared with a DMSO blank. Worms are killed for control purposes by heating in PBS at 100°C for 10 min.

Previous studies have demonstrated the utility of MTT-formazan colorimetry in proliferation and cytotoxicity assays in anti-cancer chemotherapy. Subsequently it has been demonstrated that the application of this assay was successful to determine filarial viability and for *in vitro* anti-filarial drug screening. MTT is pale-yellow in solution but when incubated with living cells is reduced by active mitochondria to yield a dark blue crystalline deposit (formazan) within cells, which once solubilized can be quantified colorimetrically.

In accordance with the present invention, MTT assays are performed to determine the effects of anti-tubulin monoclonal antibodies on the viability of parasitic nematodes. Viable control female *B. pahangi* showed rates of formazan formation that are maximal and linear during the first 30 min of the incubation with MTT. By one hour rate of formazan formation had begun to decline and plateaued between 60-90 min. Heat-killed worms show only background levels of formazan formation.

Worms treated with anti-*B. pahangi* monoclonal antibody P3D alone and in synergy with MBZ show a detectable decrease in motility 12 hours post-treatment. The other anti-*B. pahangi* monoclonal antibody 1B6 alone and in synergy with MBZ, also exhibit an apparent decline in the motility of worms, however, no mortalities are observed using these monoclonal antibodies during the experiment. No noticeable reduction is observed in the motility of the worms treated with MBZ alone or anti-chick brain monoclonal antibody 357 alone or in synergy with MBZ or the control worms, during the period the worms are in culture. From these observations, it is suggested that the reduction in the worm motility is caused mainly by the anti-*B. pahangi* monoclonal antibody alone, since MBZ alone do not have any effect on the motility of the worms.

Analysis of MTT assays demonstrates that monoclonal antibody P3D treated *B. pahangi* shows significant decline in their ability to reduce MTT to formazan (Fig. 4). This monoclonal antibody alone caused a highly significant 80% reduction in worm viability, compared with untreated live worms, 48 hours post-treatment. MBZ in synergy with monoclonal antibody P3D caused significant 70% reduction in the viability of worms. The high reduction in the viability of worms seems to be due to the presence of monoclonal antibody P3D and not MBZ. As MBZ alone induced a minimal decrease (10%) in the viability of worms. Exposure to monoclonal antibody 1B6 resulted in 40% decrease in the ability of worms to reduce MTT (Fig. 5). Monoclonal antibodies P3D and 1B6 had the same respective effects on the viability of males as for females. Anti-chick β-tubulin monoclonal antibody 357 did not show any significant effect on the viability of worms (Fig. 6). The properties of anti-*B. pahangi* and anti-chick brain monoclonal antibodies appear qualitatively similar. Differences in their inhibitory effects on the motility and viability of *B. pahangi* may depend on their different binding affinities.

Control untreated live female worms show a linear rate of formazan production and gave an absorbance reading of 1.1 at 550 nm. In contrast, heat killed worms show no ability to reduce MTT (Figs. 4 to 6). After DMSO solubilization for 1 hour the absorbance of the resulting formazan solution is determined in the presence or absence of the female worms. This is done to determine if the presence of worm had any effect on the absorbance values. In the presence of worm, there is a slight increase in the absorbance values obtained. Inhibition of MTT reduction does not always occur uniformly along the entire length of treated worms and areas retaining viability are observed. Thus by close observation of the worms during MTT reduction

it is sometimes possible to determine sites of selective damage.

Conclusion

The results of Example I demonstrate an apparent decline in the motility, when the worms are cultured with the anti-tubulin monoclonal antibodies P3D and 1B6 of the present invention. However, no noticeable reduction in the motility is observed, when the worms are treated with anti-chick monoclonal antibody 357, MBZ or IMDM/FCS culture medium without antibodies. The viability of the worms was assessed by MTT assay. The anti-*B. pahangi*, monoclonal antibodies P3D and 1B6 of the present invention, significantly reduced the viability of parasitic nematodes. No reduction in viability was observed when adult *B. pahangi* were exposed to anti-chick monoclonal antibody 357 and/or MBZ.

EXAMPLE II

Anti-parasitic antibody composition

An antibody composition to be administered to a gerbil as an anti-parasitic agent in dosage varying from 1 mg/0.5 ml to 10 mg/0.5 ml in a pharmaceutical carrier suitable for intraperitoneal administration.

The carrier for such administration is an IMDM culture media.

EXAMPLE III

Production of the eighteen amino acid peptide

The peptide consists of the amino acid sequence:

Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu
1 5 10 15

Gln Glu Glu.

To prepare the peptide for use in a vaccine, the peptide is synthesized by solid phase methodology on an Applied

Biosystems Inc. (ABI) 430A peptide synthesizer using ABI's Small Scale Rapid Cycles (SSRC) on a 0.1 mmole scale or other similar synthesizer. SSRC utilizes abbreviated single couple cycles with standard Boc chemistry. The t-Boc-L-amino acids used (1 mmole) are supplied by ABI with standard side-chain protecting groups. The completed peptide is removed from the supporting PAM (phenylacetamidomethyl) resin, concurrently with the side-chain protecting groups, by a standard HF procedure using appropriate cation scavengers (10% v/v amisole, p-cresol plus p-thiocresol, 1,4-butanedithiol plus anisole of DMS plus anisole) depending on the amino acid sequence of the peptide.

The crude peptide, after HF cleavage, is purified by preparative reverse phase chromatography on a Phenomenex C-18 Column (250 x 22.5 mm) using water acetonitrile gradients, each phase containing 0.1% TFA. The pure fractions (as determined by analytical HPLC) are pooled, acetonitrile evaporated and the aqueous solution lyophilized. The peptide is analyzed by fast atom bombardment mass spectroscopy and resulting $(M+H)^+$ is compared with the anticipated $(M+H)^+$.

EXAMPLE IV

Vaccine comprising eighteen amino acid peptide

The peptide can be prepared in vaccine dose form by well-known procedures. The vaccine can be administered sublingually, intramuscularly, subcutaneously or intranasally. For parenteral administration, such as intramuscular injection, the immunogen may be combined with a suitable carrier, for example, it may be administered in water, saline or buffered vehicles with or without various adjuvants or immunomodulating agents such as aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate (alum), beryllium sulfate, silica, kaolin, carbon, water-

in-oil emulsions, oil-in-water emulsions, muramyl dipeptide, bacterial endotoxin, lipid X, Corynebacterium parum (Propionibacterium acnes), Bordetella pertussis, polyribonucleotides, sodium alginate, lanolin, lysolecithin, vitamin A, saponin, liposomes, levamisole, DEAE-dextran, blocked copolymers or other synthetic adjuvants. Such adjuvants are available commercially from various sources, for example, Merck Adjuvant 65 (Merck and Co., Inc., Rahway, NJ).

The proportion of immunogen and adjuvant can be varied over a broad range so long as both are present in effective amounts. For example, aluminum hydroxide can be present in an amount of about 0.5% of the vaccine mixture (Al_2O_3 basis). On a per dose basis, the concentration of the immunogen can range from about 0.015 μg to about 1.5 mg per kilogram per patient body weight for an animal or human patient. A preferably dosage range in humans is about 0.1 - 1 ml, preferably about 0.1 ml. Accordingly, a dose for intramuscular injection, for example, would comprise 0.1 ml containing immunogen in admixture with 0.5% aluminum hydroxide.

The vaccine of the present invention may also be combined with other vaccines for other diseases to produce multivalent vaccines. It may also be combined with other medicaments such as antibiotics.

EXAMPLE V

Activity of B. pahangi ß-Tubulin Peptide Vaccine

The *Brugia pahangi* ß-tubulin peptide was tested for stimulation of antibody production, for suppression of adult worm burden, and for suppression of levels of microfilariae. The *B. pahangi* B-tubulin peptide was coupled to MAP using the method of Tam et. al. cited supra. These activities were tested in two different protocols. In one protocol, mongolian gerbils (jirds) were vaccinated

with the ß-tubulin peptide and then infected with *B. pahangi* larvae (vaccination -first protocol). In the other protocol, the gerbils were first infected with the larvae, then vaccinated with the ß-tubulin peptide (infection-first protocol). In each protocol, vaccination of the gerbils with vehicle alone served as a control. In each protocol, the vaccine stimulated an immune response, suppressed adult worm burden, and decreased levels of microfilariae. The infection of the mongolian gerbil with *B. pahangi* is widely accepted in the art for study of nematode infections. Ash & Rile, J. of Parasitology, 86:962 (1970).

The Protocols

In the vaccination-first protocol, the gerbils were vaccinated with the ß-tubulin peptide. Then, 14 days after the last of three injections of peptide, the gerbils were infected with *Brugia pahangi* larvae. On Day 0, the gerbils are bled and immunized by subcutaneous injection (200 micrograms per gerbil per injection) with the peptide at 1 mg/ml in a vehicle consisting of phosphate buffered saline plus Freund's Complete Adjuvant (FCA). The gerbils were again immunized on Days 14 and 28. Two weeks after the last immunization, the gerbils were infected with 100 *B. pahangi* larvae. To allow analysis of levels of microfilariae and antibody, the gerbils were bled at Days 50, 98, 154, 168, 182, 196, 210, 224, 238, and 255 post-immunization. On Day 255, adult worms were harvested from the gerbils.

In the infection-first protocol, gerbils were infected with 100 *B. pahangi*-infected larvae. Then 10 weeks later, when the infection was established, the gerbils were vaccinated three times with the peptide. On Day 0, the gerbils were bled and then injected subcutaneously with 100 infective larvae of *B. pahangi*. On Day 56, the gerbils were bled to provide a sample for

determination of the level of microfilariae and antibodies. On Days 70, 84, and 98 post-infection, the gerbils were immunized with the ß-tubulin peptide as in the vaccination-first protocol. The gerbils were then bled to provide samples for determining levels of microfilariae and antibody on Days 112, 126, 140, 156, 168, 182, 196, 210 post-infection. At Day 210, worms were harvested from the gerbils.

Antibodies were measured by ELISA conducted by standard methods using the peptide as the immobilized antigen. Microfilariae were determined according to Ash and Orihel in Parasites: A Guide to Laboratory Procedures and Identification, ASCP Press, Chicago, IL at page 99 (1987). Adult worms were determined according to Storey & Al-Mukhtar, Tropenmed. Parasit., 33:23 (1982).

Stimulation of Antibody Response

Vaccination with the *B. pahangi* ß-tubulin peptide stimulated production of anti-peptide antibodies in both the vaccination-first and infection first protocols. The results of ELISA assays are shown in Tables 1 and 2. It can be seen that the peptide stimulated an antibody response in both vaccination-first and infection-first protocols. An antibody response to the peptide was also detected in infected but not vaccinated animals. Thus, these results indicate that the peptide can stimulate an immune response both when vaccinated before and after infection.

TABLE 1

Induction of anti-ß-tubulin peptide antibodies by the ß-tubulin peptide vaccine in the vaccination-first protocol. Both the peptide vaccine group and the control group were infected with B. pahangi two weeks after the last immunization. Each ELISA test represents the mean ± std. error of sera from nine mongolian gerbils.

Days after immunization	infection	Peptide Vaccine ELISA Response[b]	Control ELISAResponse[b]
0[a]	---	0.4 ± 0.0	0.4 ± 0.0
50	7 (1 week)	2.1 ± 0.0	1.1 ± 0.0
98	56 (8 weeks)	1.9 ± 0.2	1.6 ± 0.1
154	112 (16 weeks)	1.1 ± 0.0	1.0 ± 0.1
168	126 (18 weeks)	1.5 ± 0.1	1.3 ± 0.0

[a] Pre-immunization, pre-infection sera.
[b] Optical density produced in ELISA

TABLE 2

Induction of anti-ß-tubulin peptide antibodies by the ß-tubulin peptide vaccine in the infection-first protocol. The peptide vaccine group was vaccinated 70 days after infection. Each ELISA test represents mean ± std. error of sera from nine mongolian gerbils.

Days after immunization	infection	Peptide Vaccine ELISAResponse[b]	Control ELISAResponse[b]
0[a]	--	0.3 ± 0.0	0.3 ± 0.0
112 (16 weeks)	42	2.1 ± 0.0	1.9 ± 0.0
126 (18 weeks)	56	2.3 ± 0.2	1.8 ± 0.1
140 (20 weeks)	70	2.0 ± 0.0	2.0 ± 0.0
154 (22 weeks)	74	2.2 ± 0.1	2.0 ± 0.1

[a] Pre-immunization, pre-infection sera.
[b] Optical density produced in ELISA

Reduction of Burden of Adult Worms

In both the vaccination-first and infection-first protocols, the ß-tubulin peptide vaccine reduces the adult worm load. The results are shown in Table 3. In both protocols, the peptide vaccine was effective in reducing the number of adult *B. pahangi* recovered from the gerbils by about 25% (Table 3). The vaccine was equally effective against both male and female worms, regardless of whether the vaccine was administered before or after the gerbils were infected.

TABLE 3

Adult worm burden in infected and control gerbils at the end of the protocol.

	Number of Adult Worms*		
	Male	Female	Total
Vaccination-first Protocol			
Peptide Vaccine	10 ± 2	11 ± 2	21 ± 3
Control	13 ± 2	15 ± 2	28 ± 3
Infection-first Protocol			
Peptide Vaccine	9 ± 1	9 ± 2	18 ± 2
Control	12 ± 1	13 ± 1	25 ± 3

* Mean ± std error for 9 gerbils

Reduction of Burden of Microfilariae

The ß-tubulin peptide vaccine is very efficacious against the microfilarial form of the parasite. The results are shown in Tables 4 and 5. This form of the

parasite causes symptoms in filarial and other diseases and is required for transmission of the parasite to the non-mammalian vector. This property makes the peptide vaccine useful for prophylaxis and treatment of diseases such as onchocerciasis (river blindness) in which symptoms are associated with microfilariae (Greene, J. Infect. Diseases, 166:15 (1992)). The peptide vaccine was effective in reducing the level of microfilariae by about 95% or greater in the vaccination-first protocol and by about 85% or greater in the infection-first protocol. Thus, these results indicate vaccination with the peptide is very effective for controlling microfilariae load both before and after infection.

TABLE 4

Reduction of the level of microfilariae in the vaccination-first protocol.

Weeks after Infection	Weeks after Immunization	Number of Microfilariae in Vaccinated Gerbils*	Number of Microfilariae in Control Gerbils*
16	22	3 ± 1	58 ± 13
18	24	7 ± 2	193 ± 37
20	26	6 ± 2	105 ± 25
22	28	0.5 ± 0.3	149 ± 21
24	30	5 ± 2	214 ± 28
26	32	8 ± 3	270 ± 61
28	34	2 ± 1	276 ± 60
30	36	4 ± 2	159 ± 20

* Mean ± std. error for number of microfilariae in 20 μl of blood.

TABLE 5

Reduction of the level of microfilariae in the infection-first protocol.

Weeks after Infection	Weeks after Immunization	Vaccinated Gerbils[a]	Number of Microfilariae in Control Gerbils[a]
16	2	6 ± 2	167 ± 24
18	4	18 ± 5.7	309 ± 39
20	6	76 ± 15	461 ± 63
22	8	65 ± 18	444 ± 23
24	10	31 ± 10	410 ± 51
26	12	9 ± 3	241 ± 23
28	14	32 ± 13	334 ± 39
30	16	76 ± 18	1150 ± 331

[a] Mean ± std. error for number of microfilariae in 20 μl of blood.

EXAMPLE VI

Sequencing of β-tubulins from *Dirofilariae immitis* and *Onchocerca volvulus*

Characterization of cDNA for *D. immitis* β-tubulin

cDNA sequences encoding β-tubulins from *D. immitis* and *Onchocerca volvulus* were obtained and sequenced. Adult specimens of *D. immitis* were obtained from Drs. L. Kaiser and J.F. Williams (Michigan State University, East Lansing, MI) from naturally infected dogs. Parasites were frozen at -70° following recovery and were shipped to Kalamazoo while frozen. Intact female parasites were broken under liquid nitrogen with a mortar and pestle, and RNA isolated as described previously (Klein et al., 1991). Poly A+RNA was obtained by selection with oligo (dT) cellulose (Pharmacia, NJ) by standard methods (Sambrook et al., 1989). An aliquot of poly A+RNA was sent to Stratagene, Inc. (LaJolla, CA) for construction of a cDNA library in the vector λZAP II. The phage library contained >94% inserts, size>400 bp, with a titer of $1x10^{10}$ pfu/ml after amplification according to the manufacturer.

Library screening

The phage library was plated using *E. coli* strain XL-1 Blue and screened by hybridization with a [^{32}P]-labelled cDNA encoding the 5' end of β12-16, a βtubulin gene from *Haemonchus contortus* (Geary et al., 1992, cited supra). The probe consisted of a 1 kb fragment of this cDNA, generated by cleavage of a pBluescript plasmid with *Eco*RI and *Sph*I (Geary et al., 1992, cited supra). 1x10^6 pfu were included in the primary screen, which was performed at 65°in a solution containing 1 M NaCl, 1% SDS and 10% dextran sulfate. Filters were washed in 2xSSC at 65°, and 10 positive plaques were picked. Plaque purification was done by repeated hybridization analysis, in which the wash conditions progressed in stringency to 0.2xSSC at the quaternary screen. Eight of the 10 initial plaques were pure after 4 amplifications. *In vivo* excision was performed on these clones as described by Short et al., Nucleic Acids Res., 16:7583, 1988; Klein et al., Mol. Biochem. Parasitol., 48:17, 1991). Plasmid DNA was purified by standard methods (Sambrook et al., 1989) and digested with EcoRI for restriction mapping of the inserts. Three patterns of restriction fragments were observed. However, nucleotide sequence analysis revealed that all the clones had identical inserts that varied only in size. The clone with the longest insert was subjected to nucleotide sequence analysis on both strands.

cDNA sequencing

The dideoxynucleotide chain termination reaction (Sanger et al., PNAS, 74:5463, 1977) was used as modified (Klein et al., Curr. Genetics, 16:145, 1989) to determine the sequences of *D. immitis* β-tubulin cDNAs. A Sequenase kit for double-stranded DNA sequencing was used per the manufacturers directions (United States Biochemical Corp., Cleveland, OH). Analyses of nucleotide sequences were

performed using a VAX computer and a DNA software package available from the University of Wisconsin Genetics Computer Group (Devereaux et al., Nucleic Acids, 12:387, 1984). DNA sequences for comparison were obtained from GenBank (release 59 and 65); protein sequence comparisons utilized the BESTFIT and GAP programs and FASTP algorithm (Lipman and Pearson, Science, 257:1435, 1985).

The nucleotide sequence of a cDNA spanning the coding region of *D. immitis* β-tubulin is shown in Figure 7. Included for comparison are nucleotides that differ from this sequence in an *O. volvulus* cDNA encoding the same protein and a published β-tubulin cDNA from *B. pahangi*. The predicted amino acid sequence of the *D. immitis* clone is shown in Figure 8, along with residues that differ in the β-tubulins from the other two filarial parasites. It should be noted that partial sequence obtained from other *D. immitis* and *O. volvulus* clones did not reveal allelic variance at any residues, though a complete sequence was not obtained from any other clone. In addition, though the cloning strategy employed an initial screen with a heterologous probe, only one β-tubulin isotype was found in both *D. immitis* and *O. volvulus*.

Onchocerca volvulus β-tubulin

A library prepared from adult female *O. volvulus* (Touboro, Cameroon region) was obtained from the American Type Culture Collection (Rockville, MD) under catalog #37509 (see Donelson et al., Mol. Biochem. Parasitol., 31:241, 1988 for description). The library is in λgt11, and was plated for screening on *E. coli* strain Y1090. The library was screened, and positive plaques purified and analyzed, as described above. Nucleotide sequence was obtained from 2 of 6 phage that contained the largest inserts. This analysis showed that both were truncated at the 5' end. Polymerase chain reaction experiments, using

primers derived from phage sequences and the 3' end of the sequenced *O. volvulus* β-tubulin clones revealed a similar truncation in the 4 remaining purified phage inserts.

The library was rescreened at high stringency with a probe derived from the 5' end of *D. immitis* β-tubulin. This fragment was obtained by digestion of the *D. immitis* cDNA with PstI and HindIII, and extended to approximately amino acid 100 in the sequence (see Figure 1). Conditions for screening and purifying positive plaques were as described above. From this experiment, 2 identical phage were isolated that contained the remainder of a β-tubulin open reading frame and sequence 3' to this that was identical to the previously analyzed clones.

The cDNA sequence for *O. volvulus* compared to *B. pahangi* and *D. immitis* is shown in Figure 7. The predicted amino acid sequence is shown in Figure 8. These comparisons show that the 18 amino acid peptide at the carboxy terminus is highly conserved among different genuses of *Filarioidea* and, therefore, could serve as a valuable vaccine.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modification and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows from the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Prichard, Roger K.
Bughio, Nasreen I.
Faubert, Gaetan M.
Geary, Timothy G.

(ii) TITLE OF INVENTION: Peptides of Nematode Tubulin and Methods of Use

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Merchant & Gould
(B) STREET: 90 South 7th Street, 3100 Norwest Center
(C) CITY: Minneapolis
(D) STATE: MN
(E) COUNTRY: USA
(F) ZIP: 55402

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/420,982
(B) FILING DATE: 10-APR-1995
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kowalchyk, Katherine M.
(B) REGISTRATION NUMBER: 36,848
(C) REFERENCE/DOCKET NUMBER: 10564.2US01

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 612-332-5300
(B) TELEFAX: 612-332-9081

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln
1 5 10 15

Glu Glu

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asp Glu Asp Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln
1 5 10 15

Glu Glu

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asp Asp Glu Ala Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln
1 5 10 15

Glu Glu

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1347 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1344

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
ATG AGA GAA ATA GTT CAC GTT CAA GCT GGT CAG TGT GGC AAT CAA ATT        48
Met Arg Glu Ile Val His Val Gln Ala Gly Gln Cys Gly Asn Gln Ile
  1               5                  10                  15

GGT GCC AAG TTC TGG GAA GTA ATA TCG GAT GAG CAT GGC ATT CAG CCT        96
Gly Ala Lys Phe Trp Glu Val Ile Ser Asp Glu His Gly Ile Gln Pro
            20                  25                  30

GAT GGT ACG TAT AAA GGT GAT TCA GAT TTG CAA ATT GAA CGA ATC AAT       144
Asp Gly Thr Tyr Lys Gly Asp Ser Asp Leu Gln Ile Glu Arg Ile Asn
        35                  40                  45

GTC TAC TAT AAC GAA GCA AAT GGT GGC AAA TAT GTA CCA CGA GCG ATC       192
Val Tyr Tyr Asn Glu Ala Asn Gly Gly Lys Tyr Val Pro Arg Ala Ile
    50                  55                  60

CTT GTC GAT CTG GAA CCT GGT ACT ATG GAT TCT ATT CGA GGA GGT GGA       240
Leu Val Asp Leu Glu Pro Gly Thr Met Asp Ser Ile Arg Gly Gly Gly
65                  70                  75                  80

TTT GGT CAA CTG TTC CGA CCG GAT AAT TTC GTA TTT GGA CAG AGT GGA       288
Phe Gly Gln Leu Phe Arg Pro Asp Asn Phe Val Phe Gly Gln Ser Gly
                85                  90                  95

GCT GGC AAC AAC TGG GCT AAG GGA CAT TAC ACA GAA GGT GCC GAA TTA       336
Ala Gly Asn Asn Trp Ala Lys Gly His Tyr Thr Glu Gly Ala Glu Leu
            100                 105                 110

GTT GAT AAC GTT TTG GAC GTA ATA CGA AAA GAA GCT GAA GGA TGC GAC       384
Val Asp Asn Val Leu Asp Val Ile Arg Lys Glu Ala Glu Gly Cys Asp
        115                 120                 125

TGT CTT CAG GGA TTC CAA CTG ACT CAT TCA CTT GGA GGT GGT ACA GGT       432
Cys Leu Gln Gly Phe Gln Leu Thr His Ser Leu Gly Gly Gly Thr Gly
    130                 135                 140

TCT GGT ATG GGA ACA TTG CTT ATC TCG AAG ATC CGT GAG GAA TAT CCA       480
Ser Gly Met Gly Thr Leu Leu Ile Ser Lys Ile Arg Glu Glu Tyr Pro
145                 150                 155                 160

GAT CGG ATT ATG AGC TCT TTT TCG GTT GTG CCA TCA CCT AAA GTA TCA       528
Asp Arg Ile Met Ser Ser Phe Ser Val Val Pro Ser Pro Lys Val Ser
                165                 170                 175
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```
GAT GTT GTG TTG GAA CCT TAC AAT GCA ACG TTA TCA GTG CAT CAA TTA          576
Asp Val Val Leu Glu Pro Tyr Asn Ala Thr Leu Ser Val His Gln Leu
            180                 185                 190

GTT GAA AAC ACT GAT GAA ACT TTC TGC ATT GAT AAT GAA GCT TTA TAT          624
Val Glu Asn Thr Asp Glu Thr Phe Cys Ile Asp Asn Glu Ala Leu Tyr
        195                 200                 205

GAT ATC TGC TTC CGA ACA TTG AAA TTG ACG AAT CCA ACT TAC GGC GAT          672
Asp Ile Cys Phe Arg Thr Leu Lys Leu Thr Asn Pro Thr Tyr Gly Asp
    210                 215                 220

CTC AAT CAC TTG GTA TCT GTA ACA ATG TCT GGA GTA ACA ACA TGT TTA          720
Leu Asn His Leu Val Ser Val Thr Met Ser Gly Val Thr Thr Cys Leu
225                 230                 235                 240

CGT TTC CCT GGA CAA TTA AAT GCC GAT CTT CGT AAG CTT GCT GTT AAT          768
Arg Phe Pro Gly Gln Leu Asn Ala Asp Leu Arg Lys Leu Ala Val Asn
                245                 250                 255

ATG GTA CCA TTC CCA CGT TTG CAT TTC TTC ATG CCT GGA TTT GCT CCT          816
Met Val Pro Phe Pro Arg Leu His Phe Phe Met Pro Gly Phe Ala Pro
            260                 265                 270

CTC TCT GCA CGT GGC GCT GCT GCT TAT CGG GCA CTC AAT GTT GCT GAG          864
Leu Ser Ala Arg Gly Ala Ala Ala Tyr Arg Ala Leu Asn Val Ala Glu
        275                 280                 285

CTC ACT CAA CAG ATG TTT GAT GCC AAA AAT ATG ATG GCA GCA TGT GAT          912
Leu Thr Gln Gln Met Phe Asp Ala Lys Asn Met Met Ala Ala Cys Asp
    290                 295                 300

CCA CGT CAT GGC CGT TAT CTG ACC GTA GCT GCT ATG TTC CGA GGC AGA          960
Pro Arg His Gly Arg Tyr Leu Thr Val Ala Ala Met Phe Arg Gly Arg
305                 310                 315                 320

ATG TCG ATG CGA GAA GTA GAC GAG CAA ATG ATG CAA GTG CAG AAT AAG         1008
Met Ser Met Arg Glu Val Asp Glu Gln Met Met Gln Val Gln Asn Lys
                325                 330                 335

AAT TCA TCG TAT TTC GTT GAA TGG ATT CCG AAT AAC GTA AAA ACA GCT         1056
Asn Ser Ser Tyr Phe Val Glu Trp Ile Pro Asn Asn Val Lys Thr Ala
            340                 345                 350

GTT TGC GAT ATT CCA CCA CGT GGC TTG AAG ATG AGC GCA ACA TTC ATC         1104
Val Cys Asp Ile Pro Pro Arg Gly Leu Lys Met Ser Ala Thr Phe Ile
        355                 360                 365

GGC AAT ACA ACA GCC ATA CAA GAA CTT TTT AAA CGC ATT TCT GAA CAG         1152
Gly Asn Thr Thr Ala Ile Gln Glu Leu Phe Lys Arg Ile Ser Glu Gln
    370                 375                 380

TTT ACT GCT ATG TTC CGA CGT AAA GCA TTC TTG CAT TGG TAT ACT GGA         1200
Phe Thr Ala Met Phe Arg Arg Lys Ala Phe Leu His Trp Tyr Thr Gly
385                 390                 395                 400

GAA GGT ATG GAT GAA ATG GAA TTC ACG GAA GCA GAG AGT AAC ATG AAT         1248
Glu Gly Met Asp Glu Met Glu Phe Thr Glu Ala Glu Ser Asn Met Asn
                405                 410                 415
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GAC TTG GTG TCT GAA TAT CAG CAA TAT CAG GAT GCA ACG TCT GAT GAA      1296
Asp Leu Val Ser Glu Tyr Gln Gln Tyr Gln Asp Ala Thr Ser Asp Glu
            420                 425                 430

GAC GGT GAT CTT CAG GAA GGT GAA TCG GAA TAT ATT GAG CAA GAG GAA      1344
Asp Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln Glu Glu
        435                 440                 445

TAA                                                                  1347
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 448 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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Met Arg Glu Ile Val His Val Gln Ala Gly Gln Cys Gly Asn Gln Ile
 1               5                  10                  15

Gly Ala Lys Phe Trp Glu Val Ile Ser Asp Glu His Gly Ile Gln Pro
            20                  25                  30

Asp Gly Thr Tyr Lys Gly Asp Ser Asp Leu Gln Ile Glu Arg Ile Asn
        35                  40                  45

Val Tyr Tyr Asn Glu Ala Asn Gly Gly Lys Tyr Val Pro Arg Ala Ile
    50                  55                  60

Leu Val Asp Leu Glu Pro Gly Thr Met Asp Ser Ile Arg Gly Gly Gly
65                  70                  75                  80

Phe Gly Gln Leu Phe Arg Pro Asp Asn Phe Val Phe Gly Gln Ser Gly
                85                  90                  95

Ala Gly Asn Asn Trp Ala Lys Gly His Tyr Thr Glu Gly Ala Glu Leu
            100                 105                 110

Val Asp Asn Val Leu Asp Val Ile Arg Lys Glu Ala Glu Gly Cys Asp
        115                 120                 125

Cys Leu Gln Gly Phe Gln Leu Thr His Ser Leu Gly Gly Gly Thr Gly
    130                 135                 140

Ser Gly Met Gly Thr Leu Leu Ile Ser Lys Ile Arg Glu Glu Tyr Pro
145                 150                 155                 160

Asp Arg Ile Met Ser Ser Phe Ser Val Val Pro Ser Pro Lys Val Ser
                165                 170                 175

Asp Val Val Leu Glu Pro Tyr Asn Ala Thr Leu Ser Val His Gln Leu
            180                 185                 190
```

Val Glu Asn Thr Asp Glu Thr Phe Cys Ile Asp Asn Glu Ala Leu Tyr
195 200 205

Asp Ile Cys Phe Arg Thr Leu Lys Leu Thr Asn Pro Thr Tyr Gly Asp
210 215 220

Leu Asn His Leu Val Ser Val Thr Met Ser Gly Val Thr Thr Cys Leu
225 230 235 240

Arg Phe Pro Gly Gln Leu Asn Ala Asp Leu Arg Lys Leu Ala Val Asn
245 250 255

Met Val Pro Phe Pro Arg Leu His Phe Phe Met Pro Gly Phe Ala Pro
260 265 270

Leu Ser Ala Arg Gly Ala Ala Ala Tyr Arg Ala Leu Asn Val Ala Glu
275 280 285

Leu Thr Gln Gln Met Phe Asp Ala Lys Asn Met Met Ala Ala Cys Asp
290 295 300

Pro Arg His Gly Arg Tyr Leu Thr Val Ala Ala Met Phe Arg Gly Arg
305 310 315 320

Met Ser Met Arg Glu Val Asp Glu Gln Met Met Gln Val Gln Asn Lys
325 330 335

Asn Ser Ser Tyr Phe Val Glu Trp Ile Pro Asn Asn Val Lys Thr Ala
340 345 350

Val Cys Asp Ile Pro Pro Arg Gly Leu Lys Met Ser Ala Thr Phe Ile
355 360 365

Gly Asn Thr Thr Ala Ile Gln Glu Leu Phe Lys Arg Ile Ser Glu Gln
370 375 380

Phe Thr Ala Met Phe Arg Arg Lys Ala Phe Leu His Trp Tyr Thr Gly
385 390 395 400

Glu Gly Met Asp Glu Met Glu Phe Thr Glu Ala Glu Ser Asn Met Asn
405 410 415

Asp Leu Val Ser Glu Tyr Gln Gln Tyr Gln Asp Ala Thr Ser Asp Glu
420 425 430

Asp Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln Glu Glu
435 440 445

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1347 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1344

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
ATG AGA GAA ATT GTT CAT GTT CAA GCT GGT CAA TGT GGC AAT CAA ATT        48
Met Arg Glu Ile Val His Val Gln Ala Gly Gln Cys Gly Asn Gln Ile
 1               5                  10                  15

GGT GCC AAG TTC TGG GAA GTA ATA TCG GAT GAA CAT GGC ATT CAA CCT        96
Gly Ala Lys Phe Trp Glu Val Ile Ser Asp Glu His Gly Ile Gln Pro
            20                  25                  30

GAT GGT ACT TAT AAA GGT GAT TCA GAT TTG CAA ATT GAG CGA ATC AAT       144
Asp Gly Thr Tyr Lys Gly Asp Ser Asp Leu Gln Ile Glu Arg Ile Asn
        35                  40                  45

GTC TAC TAT AAT GAA GCG AAT GGT GGC GAA TAT GTA CCA CGA GCA ATC       192
Val Tyr Tyr Asn Glu Ala Asn Gly Gly Glu Tyr Val Pro Arg Ala Ile
    50                  55                  60

CTT GTC GAT CTG GAA CCG GGT ACT ATG GAT TCC ATT CGA GGA GGT GGA       240
Leu Val Asp Leu Glu Pro Gly Thr Met Asp Ser Ile Arg Gly Gly Gly
65                  70                  75                  80

TTT GGC CAA CTG TTC CGA CCG GAC AAT TTT GTA TTT GGA CAA AGT GGA       288
Phe Gly Gln Leu Phe Arg Pro Asp Asn Phe Val Phe Gly Gln Ser Gly
                85                  90                  95

GCT GGC AAC AAT TGG GCT AAG GGA CAT TAC ACG GAA GGT GCA GAA TTG       336
Ala Gly Asn Asn Trp Ala Lys Gly His Tyr Thr Glu Gly Ala Glu Leu
            100                 105                 110

GTT GAT AAT GTA TTG GAT GTA ATA CGA AAA GAG GCT GAA GGA TGC GAC       384
Val Asp Asn Val Leu Asp Val Ile Arg Lys Glu Ala Glu Gly Cys Asp
        115                 120                 125

TGT CTT CAG GGA TTT CAA TTG ACT CAT TCA CTT GGC GGT GGT ACC GGT       432
Cys Leu Gln Gly Phe Gln Leu Thr His Ser Leu Gly Gly Gly Thr Gly
    130                 135                 140

TCT GGT ATG GGA ACA TTG TTG ATC TCG AAA ATT CGT GAG GAA TAT CCG       480
Ser Gly Met Gly Thr Leu Leu Ile Ser Lys Ile Arg Glu Glu Tyr Pro
145                 150                 155                 160
```

```
GAT CGA ATT ATG AGC TCT TTT TCG GTT GTA CCA TCA CCC AAA GTA TCA        528
Asp Arg Ile Met Ser Ser Phe Ser Val Val Pro Ser Pro Lys Val Ser
                165                 170                 175

GAT GTT GTA TTG GAA CCT TAT AAC GCA ACA TTA TCA GTG CAT CAA TTA        576
Asp Val Val Leu Glu Pro Tyr Asn Ala Thr Leu Ser Val His Gln Leu
            180                 185                 190

GTT GAA AAC ACT GAC GAA ACT TTC TGC ATT GAT AAC GAA GCT TTA TAT        624
Val Glu Asn Thr Asp Glu Thr Phe Cys Ile Asp Asn Glu Ala Leu Tyr
        195                 200                 205

GAC ATC TGC TTC CGA ACA TTG AAA TTG ACG AAT CCA ACT TAT GGC GAT        672
Asp Ile Cys Phe Arg Thr Leu Lys Leu Thr Asn Pro Thr Tyr Gly Asp
    210                 215                 220

CTC AAT CAT TTG GTA TCT GTG ACA ATG TCT GGA GTG ACA ACA TGC TTG        720
Leu Asn His Leu Val Ser Val Thr Met Ser Gly Val Thr Thr Cys Leu
225                 230                 235                 240

CGT TTT CCT GGA CAG TTA AAT GCC GAT CTT CGT AAG CTC GCT GTT AAT        768
Arg Phe Pro Gly Gln Leu Asn Ala Asp Leu Arg Lys Leu Ala Val Asn
                245                 250                 255

ATG GTA CCA TTC CCA CGA TTG CAT TTC TTC ATG CCA GGA TTT GCT CCT        816
Met Val Pro Phe Pro Arg Leu His Phe Phe Met Pro Gly Phe Ala Pro
            260                 265                 270

CTC TCT GCT CGT GGT GCT GCT GCT TAT CGG GCA CTT AAT GTT GCT GAG        864
Leu Ser Ala Arg Gly Ala Ala Ala Tyr Arg Ala Leu Asn Val Ala Glu
        275                 280                 285

CTT ACT CAA CAG ATG TTT GAC GCC AAA AAT ATG ATG GCA GCA TGT GAT        912
Leu Thr Gln Gln Met Phe Asp Ala Lys Asn Met Met Ala Ala Cys Asp
    290                 295                 300

CCA CGT CAT GGC CGT TAC TTA ACC GTA GCC GCT ATG TTC CGA GGC AGA        960
Pro Arg His Gly Arg Tyr Leu Thr Val Ala Ala Met Phe Arg Gly Arg
305                 310                 315                 320

ATG TCG ATG CGA GAA GTG GAT GAG CAA ATG ATG CAA GTG CAG AAT AAG       1008
Met Ser Met Arg Glu Val Asp Glu Gln Met Met Gln Val Gln Asn Lys
                325                 330                 335

AAT TCA TCG TAC TTT GTT GAA TGG ATT CCA AAT AAC GTA AAA ACA GCA       1056
Asn Ser Ser Tyr Phe Val Glu Trp Ile Pro Asn Asn Val Lys Thr Ala
            340                 345                 350

GTT TGC GAT ATT CCA CCA CGT GGG TTG AAG ATG AGC GCA ACA TTC ATC       1104
Val Cys Asp Ile Pro Pro Arg Gly Leu Lys Met Ser Ala Thr Phe Ile
        355                 360                 365

GGA AAT ACA ACA GCC ATA CAA GAA CTT TTC AAA CGC ATT TCT GAG CAG       1152
Gly Asn Thr Thr Ala Ile Gln Glu Leu Phe Lys Arg Ile Ser Glu Gln
    370                 375                 380

TTC ACT GCC ATG TTC CGA CGT AAA GCA TTC TTG CAT TGG TAT ACT GGA       1200
Phe Thr Ala Met Phe Arg Arg Lys Ala Phe Leu His Trp Tyr Thr Gly
385                 390                 395                 400
```

```
GAA GGT ATG GAT GAA ATG GAA TTC ACT GAA GCA GAA AGT AAC ATG AAT      1248
Glu Gly Met Asp Glu Met Glu Phe Thr Glu Ala Glu Ser Asn Met Asn
                405                 410                 415

GAT TTG GTT TCT GAA TAT CAG CAG TAT CAA GAT GCA ACG GCT GAT GAT      1296
Asp Leu Val Ser Glu Tyr Gln Gln Tyr Gln Asp Ala Thr Ala Asp Asp
            420                 425                 430

GAA GCT GAT CTT CAG GAA GGT GAA TCG GAA TAC ATT GAA CAG GAA GAG      1344
Glu Ala Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln Glu Glu
        435                 440                 445

TAA                                                                 1347
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 448 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Met Arg Glu Ile Val His Val Gln Ala Gly Gln Cys Gly Asn Gln Ile
  1               5                  10                  15

Gly Ala Lys Phe Trp Glu Val Ile Ser Asp Glu His Gly Ile Gln Pro
            20                  25                  30

Asp Gly Thr Tyr Lys Gly Asp Ser Asp Leu Gln Ile Glu Arg Ile Asn
        35                  40                  45

Val Tyr Tyr Asn Glu Ala Asn Gly Gly Glu Tyr Val Pro Arg Ala Ile
    50                  55                  60

Leu Val Asp Leu Glu Pro Gly Thr Met Asp Ser Ile Arg Gly Gly Gly
65                  70                  75                  80

Phe Gly Gln Leu Phe Arg Pro Asp Asn Phe Val Phe Gly Gln Ser Gly
                85                  90                  95

Ala Gly Asn Asn Trp Ala Lys Gly His Tyr Thr Glu Gly Ala Glu Leu
            100                 105                 110

Val Asp Asn Val Leu Asp Val Ile Arg Lys Glu Ala Glu Gly Cys Asp
        115                 120                 125

Cys Leu Gln Gly Phe Gln Leu Thr His Ser Leu Gly Gly Gly Thr Gly
    130                 135                 140

Ser Gly Met Gly Thr Leu Leu Ile Ser Lys Ile Arg Glu Glu Tyr Pro
145                 150                 155                 160

Asp Arg Ile Met Ser Ser Phe Ser Val Val Pro Ser Pro Lys Val Ser
                165                 170                 175
```

```
Asp Val Val Leu Glu Pro Tyr Asn Ala Thr Leu Ser Val His Gln Leu
            180                 185                 190

Val Glu Asn Thr Asp Glu Thr Phe Cys Ile Asp Asn Glu Ala Leu Tyr
        195                 200                 205

Asp Ile Cys Phe Arg Thr Leu Lys Leu Thr Asn Pro Thr Tyr Gly Asp
    210                 215                 220

Leu Asn His Leu Val Ser Val Thr Met Ser Gly Val Thr Thr Cys Leu
225                 230                 235                 240

Arg Phe Pro Gly Gln Leu Asn Ala Asp Leu Arg Lys Leu Ala Val Asn
                245                 250                 255

Met Val Pro Phe Pro Arg Leu His Phe Phe Met Pro Gly Phe Ala Pro
            260                 265                 270

Leu Ser Ala Arg Gly Ala Ala Ala Tyr Arg Ala Leu Asn Val Ala Glu
        275                 280                 285

Leu Thr Gln Gln Met Phe Asp Ala Lys Asn Met Met Ala Ala Cys Asp
    290                 295                 300

Pro Arg His Gly Arg Tyr Leu Thr Val Ala Ala Met Phe Arg Gly Arg
305                 310                 315                 320

Met Ser Met Arg Glu Val Asp Glu Gln Met Met Gln Val Gln Asn Lys
                325                 330                 335

Asn Ser Ser Tyr Phe Val Glu Trp Ile Pro Asn Asn Val Lys Thr Ala
            340                 345                 350

Val Cys Asp Ile Pro Pro Arg Gly Leu Lys Met Ser Ala Thr Phe Ile
        355                 360                 365

Gly Asn Thr Thr Ala Ile Gln Glu Leu Phe Lys Arg Ile Ser Glu Gln
    370                 375                 380

Phe Thr Ala Met Phe Arg Arg Lys Ala Phe Leu His Trp Tyr Thr Gly
385                 390                 395                 400

Glu Gly Met Asp Glu Met Glu Phe Thr Glu Ala Glu Ser Asn Met Asn
                405                 410                 415

Asp Leu Val Ser Glu Tyr Gln Gln Tyr Gln Asp Ala Thr Ala Asp Asp
            420                 425                 430

Glu Ala Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln Glu Glu
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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1347 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1344

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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GGT GCC AAG TTC TGG GAA GTA ATA TCG GAT GAA CAT GGT GTT CAA CCT        96
Gly Ala Lys Phe Trp Glu Val Ile Ser Asp Glu His Gly Val Gln Pro
            20                  25                  30

GAT GGT ACA TAT AAA GGT GAT TCA GAC CTG CAA ATT GAA CGA ATC AAC       144
Asp Gly Thr Tyr Lys Gly Asp Ser Asp Leu Gln Ile Glu Arg Ile Asn
        35                  40                  45

GTC TAC TAT AAT GAA GCG AAT GGG GGC AAA TAT GTA CCA CGA GCA GTC       192
Val Tyr Tyr Asn Glu Ala Asn Gly Gly Lys Tyr Val Pro Arg Ala Val
    50                  55                  60

CTT GTT GAT TTG GAA CCA GGT ACC ATG GAT TCT ATT CGA GGA GGT GAG       240
Leu Val Asp Leu Glu Pro Gly Thr Met Asp Ser Ile Arg Gly Gly Glu
65                  70                  75                  80

TTT GGG CAA CTA TTC CGA CCT GAC AAT TTT GTT TTT GGG CAA AGT GGA       288
Phe Gly Gln Leu Phe Arg Pro Asp Asn Phe Val Phe Gly Gln Ser Gly
                85                  90                  95

GCT GGC AAC AAC TGG GCT AAG GGA CAT TAT ACG GAA GGT GCG GAA CTA       336
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            100                 105                 110

GTT GAT AAT GTG TTG GAC GTG ATA CGA AAA GAA GCT GAG GGA TGC GAT       384
Val Asp Asn Val Leu Asp Val Ile Arg Lys Glu Ala Glu Gly Cys Asp
        115                 120                 125

TGT CTT CAG GGA TTT CAA CTA ACG CAT TCA CTT GGT GGT GGT ACC GGT       432
Cys Leu Gln Gly Phe Gln Leu Thr His Ser Leu Gly Gly Gly Thr Gly
    130                 135                 140

TCC GGC ATG GGA ACA TTG CTG ATC TCG AAA ATT CGT GAG GAG TAT CCG       480
Ser Gly Met Gly Thr Leu Leu Ile Ser Lys Ile Arg Glu Glu Tyr Pro
145                 150                 155                 160
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                165                 170                 175

GAT GTT GTG TTG GAA CCC TAC AAT GCA ACA TTA TCA GTC CAC CAA CTA      576
Asp Val Val Leu Glu Pro Tyr Asn Ala Thr Leu Ser Val His Gln Leu
            180                 185                 190

GTT GAA AAC ACT GAC GAA ACT TTC TGC ATT GAT AAC GAG GCT TTG TAT      624
Val Glu Asn Thr Asp Glu Thr Phe Cys Ile Asp Asn Glu Ala Leu Tyr
        195                 200                 205

GAC ATC TGC TTC CGA ACG TTG AAG TTG GCA AAT CCA ACT TAC GGT GAC      672
Asp Ile Cys Phe Arg Thr Leu Lys Leu Ala Asn Pro Thr Tyr Gly Asp
    210                 215                 220

CTC AAC CAT TTG GTG TCT GTG ACA ATG TCG GGA GTA ACA ACT TGC TTA      720
Leu Asn His Leu Val Ser Val Thr Met Ser Gly Val Thr Thr Cys Leu
225                 230                 235                 240

CGT TTC CCT GGA CAG TTG AAC GCC GAT CTC CGT AAA CTT GCC GTC AAT      768
Arg Phe Pro Gly Gln Leu Asn Ala Asp Leu Arg Lys Leu Ala Val Asn
                245                 250                 255

ATG GTG CCA TTC CCA CGG TTG CAT TTC TTT ATG CCA GGA TTT GCT CCT      816
Met Val Pro Phe Pro Arg Leu His Phe Phe Met Pro Gly Phe Ala Pro
            260                 265                 270

CTC TCT GCT CGT GAT GCT GCT GCT TAT CGA GCC CTC AAT GTT GCT GAA      864
Leu Ser Ala Arg Asp Ala Ala Ala Tyr Arg Ala Leu Asn Val Ala Glu
        275                 280                 285

CTT ACT CAA CAG ATG TTT GAT GCC AAA AAT ATG ATG GCA GCA TGT GAT      912
Leu Thr Gln Gln Met Phe Asp Ala Lys Asn Met Met Ala Ala Cys Asp
    290                 295                 300

CCG CGT CAT GGT CGT TAC CTA ACC GTA GCT GCC ATG TTC CGA GGT AGA      960
Pro Arg His Gly Arg Tyr Leu Thr Val Ala Ala Met Phe Arg Gly Arg
305                 310                 315                 320

ATG TCT ATG CGG GAA GTA GAC GAG CAA ATG ATG CAA GTA CAG ATT AAG     1008
Met Ser Met Arg Glu Val Asp Glu Gln Met Met Gln Val Gln Ile Lys
                325                 330                 335

AAT TCA TCG TAT TTC GTT GAA TGG ATT CCA AAT AAC GTA AAG ACA GCT     1056
Asn Ser Ser Tyr Phe Val Glu Trp Ile Pro Asn Asn Val Lys Thr Ala
            340                 345                 350

GTT TGC GAC ATT CCA CCA CGT GGA TTA AAG ATG AGC GCA ACA TTT ATT     1104
Val Cys Asp Ile Pro Pro Arg Gly Leu Lys Met Ser Ala Thr Phe Ile
        355                 360                 365

GGA AAT ACA ACA GCT ATA CAA GAA CTT TTC AAG CGC ATT TCC GAA CAG     1152
Gly Asn Thr Thr Ala Ile Gln Glu Leu Phe Lys Arg Ile Ser Glu Gln
    370                 375                 380

TTT ACT GCC ATG TTC CGA CGT AAA GCA TTC TTG CAT TGG TAT ACT GGC     1200
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385                 390                 395                 400
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GAA GGT ATG GAT GAA ATG GAA TTC ACG GAA GCG GAG AGT AAT GTG AAT      1248
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                405                 410                 415

GAC TTG GTG TCC GAA TAT CAA CAA TAT CAG GAT GCG ACG GCT GAT GAA      1296
Asp Leu Val Ser Glu Tyr Gln Gln Tyr Gln Asp Ala Thr Ala Asp Glu
            420                 425                 430

GAA GGT GAT CTT CAG GAA GGT GAA TCG GAA TAC ATT GAA CAG GAA GAG      1344
Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln Glu Glu
        435                 440                 445

TGA                                                                 1347
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(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 448 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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Asp Gly Thr Tyr Lys Gly Asp Ser Asp Leu Gln Ile Glu Arg Ile Asn
        35                  40                  45

Val Tyr Tyr Asn Glu Ala Asn Gly Gly Lys Tyr Val Pro Arg Ala Val
    50                  55                  60

Leu Val Asp Leu Glu Pro Gly Thr Met Asp Ser Ile Arg Gly Gly Glu
65                  70                  75                  80

Phe Gly Gln Leu Phe Arg Pro Asp Asn Phe Val Phe Gly Gln Ser Gly
                85                  90                  95

Ala Gly Asn Asn Trp Ala Lys Gly His Tyr Thr Glu Gly Ala Glu Leu
            100                 105                 110

Val Asp Asn Val Leu Asp Val Ile Arg Lys Glu Ala Glu Gly Cys Asp
        115                 120                 125

Cys Leu Gln Gly Phe Gln Leu Thr His Ser Leu Gly Gly Gly Thr Gly
    130                 135                 140

Ser Gly Met Gly Thr Leu Leu Ile Ser Lys Ile Arg Glu Glu Tyr Pro
145                 150                 155                 160

Asp Arg Ile Met Ser Ser Phe Ser Val Val Pro Ser Pro Lys Val Ser
                165                 170                 175
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Asp Val Val Leu Glu Pro Tyr Asn Ala Thr Leu Ser Val His Gln Leu
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Val Glu Asn Thr Asp Glu Thr Phe Cys Ile Asp Asn Glu Ala Leu Tyr
195 200 205

Asp Ile Cys Phe Arg Thr Leu Lys Leu Ala Asn Pro Thr Tyr Gly Asp
210 215 220

Leu Asn His Leu Val Ser Val Thr Met Ser Gly Val Thr Thr Cys Leu
225 230 235 240

Arg Phe Pro Gly Gln Leu Asn Ala Asp Leu Arg Lys Leu Ala Val Asn
245 250 255

Met Val Pro Phe Pro Arg Leu His Phe Phe Met Pro Gly Phe Ala Pro
260 265 270

Leu Ser Ala Arg Asp Ala Ala Ala Tyr Arg Ala Leu Asn Val Ala Glu
275 280 285

Leu Thr Gln Gln Met Phe Asp Ala Lys Asn Met Met Ala Ala Cys Asp
290 295 300

Pro Arg His Gly Arg Tyr Leu Thr Val Ala Ala Met Phe Arg Gly Arg
305 310 315 320

Met Ser Met Arg Glu Val Asp Glu Gln Met Met Gln Val Gln Ile Lys
325 330 335

Asn Ser Ser Tyr Phe Val Glu Trp Ile Pro Asn Asn Val Lys Thr Ala
340 345 350

Val Cys Asp Ile Pro Pro Arg Gly Leu Lys Met Ser Ala Thr Phe Ile
355 360 365

Gly Asn Thr Thr Ala Ile Gln Glu Leu Phe Lys Arg Ile Ser Glu Gln
370 375 380

Phe Thr Ala Met Phe Arg Arg Lys Ala Phe Leu His Trp Tyr Thr Gly
385 390 395 400

Glu Gly Met Asp Glu Met Glu Phe Thr Glu Ala Glu Ser Asn Val Asn
405 410 415

Asp Leu Val Ser Glu Tyr Gln Gln Tyr Gln Asp Ala Thr Ala Asp Glu
420 425 430

Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln Glu Glu
435 440 445

WE CLAIM:

1. A vaccine for filarial parasite infection comprising at least one peptide having the sequence of amino acids at the carboxy terminal end of 0-tubulin from a filarial parasite in association with a pharmaceutical diluent.

2. A vaccine according to claim 1 wherein the peptide is attached to a carrier agent.

3. A vaccine according to claim 2 wherein the carrier agent is MAP.

4. A vaccine according to claim 1 wherein the peptide has the sequence of 0-tubulin from 13. *pahangi*.

5. A vaccine according to claim 4 wherein the peptide has an amino acid sequence:

Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu
1 5 10 15
Gln Glu Glu.

6. A vaccine according to claim 1 wherein the peptide has the sequence of 0-tubulin from *Dirofilaria immitis*.

7. A vaccine according to claim 6 wherein the peptide has an amino acid sequence:

Asp Glu Asp Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu
1 5 10 15
Gln Glu Glu.

8. A vaccine according to claim 1 wherein the peptide has the sequence of 0-tubulin from *Onchocerca volvulus*.

9. A vaccine according to claim 8 wherein the peptide has an amino acid sequence:

Asp Asp Glu Ala Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu
1 5 10 15
Gln Glu Glu.

10. A vaccine according to claim 1 wherein the vaccine further comprises an adjuvant.

11. A method of protecting an animal against filarial parasite infection comprising administering a vaccine comprising a at least one peptide that has the sequence of amino acids of the carboxy terminal end of 0-tubulin from a filarial parasite in admixture with a pharmaceutical diluent.

12. A vaccine for filarial parasite infection comprising a peptide which has the amino acid sequence:
Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu
1 5 10 15
Gln Glu Glu, or fragment thereof, in association with a pharmaceutical carrier.

13. A vaccine according to claim 12, wherein the parasite is *Wuchereria bancrofti*.

14. A vaccine according to claim 12, wherein the parasite is *Brugia malayi*.

15. A vaccine according to claim 12, wherein the vaccine reduces the worm burden.

16. A vaccine according to claim 12, wherein the vaccine reduces the microfilarial load.

17. A method of immunizing animals against filarial parasites comprising: administering a vaccine according to claim 1 to an animal.

18. A method according to claim 17, wherein the parasite is *Dirofilaria immitis* and the animal is a canine.

19. A method according to claim 17, wherein the vaccine is administered after infection.

20. A method of controlling microfilarial load in an animal infected with a filarial parasite comprising:
administering a vaccine of claim 18 to an animal.

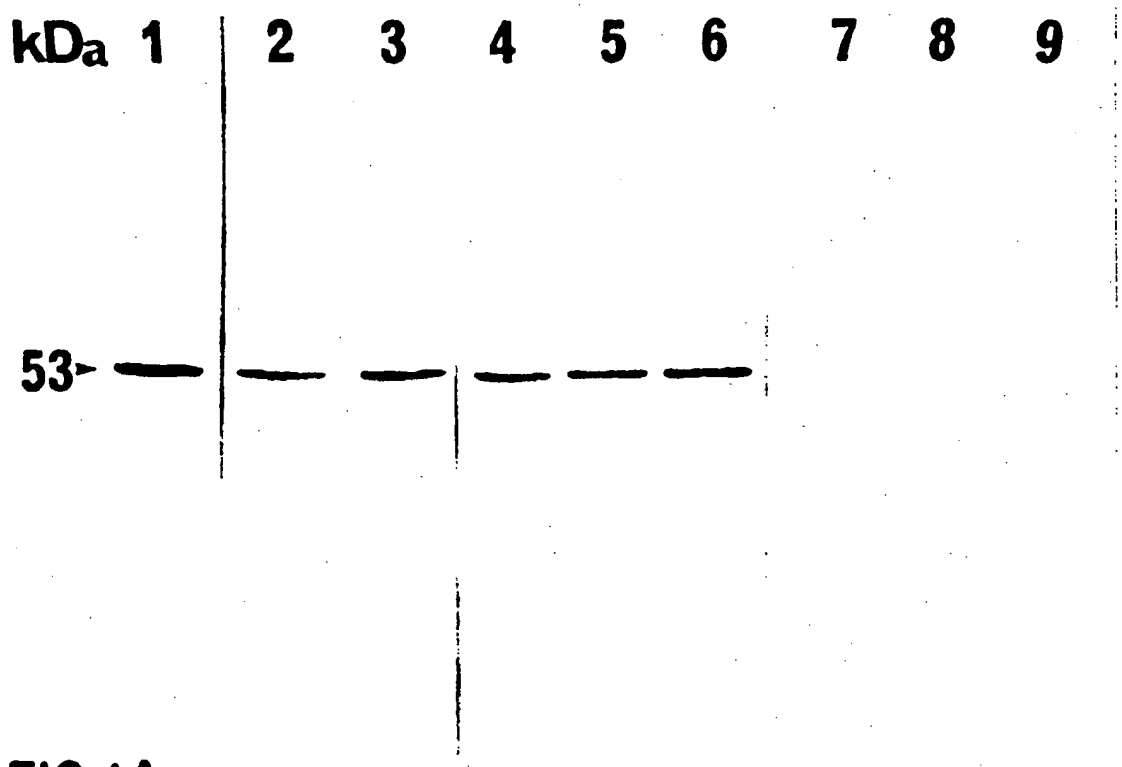


FIG. 1A

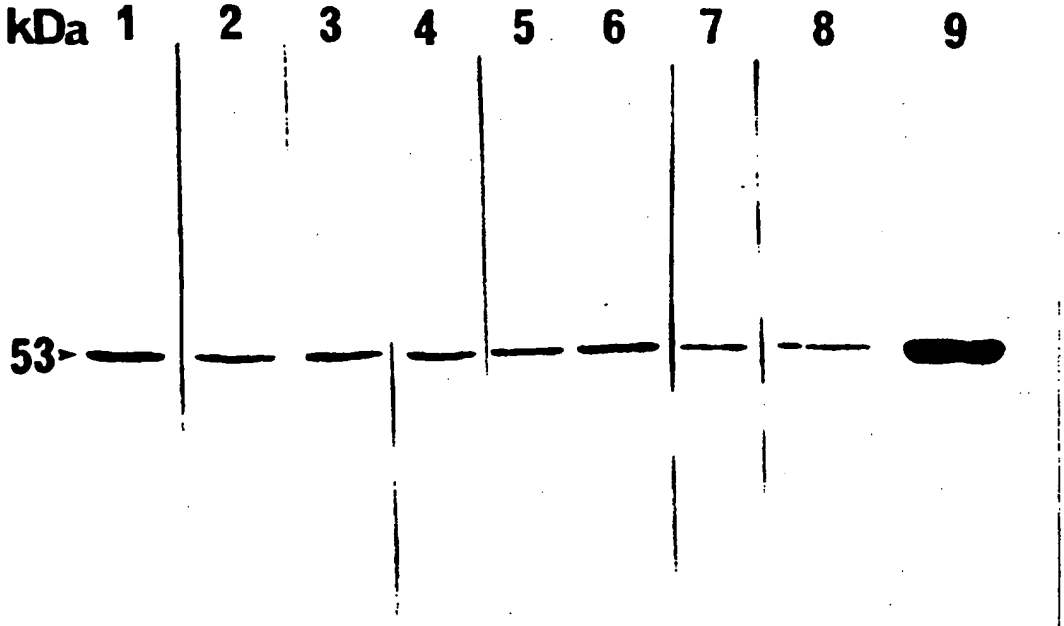


FIG. 1B

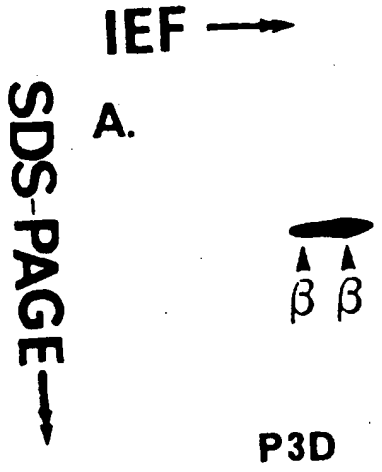


FIG. 2 A

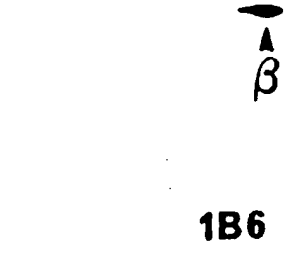


FIG. 2 B

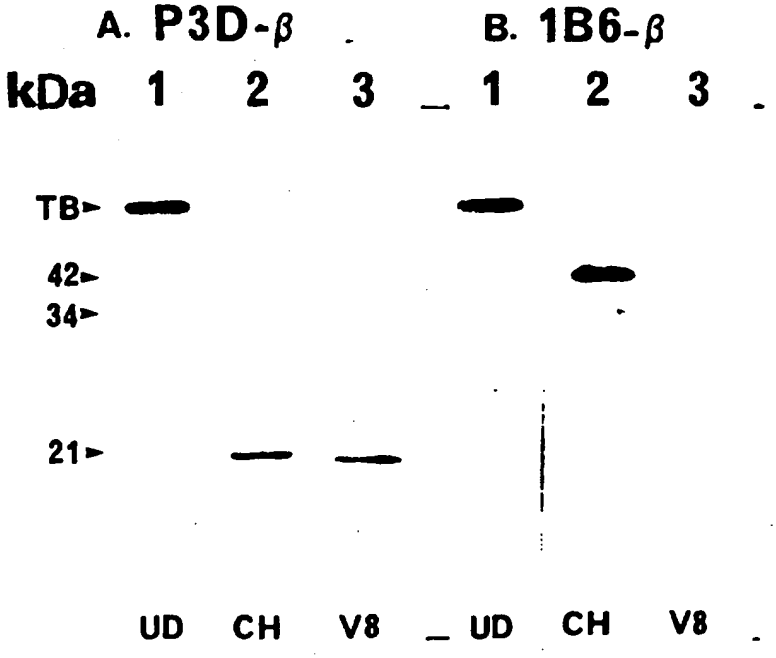
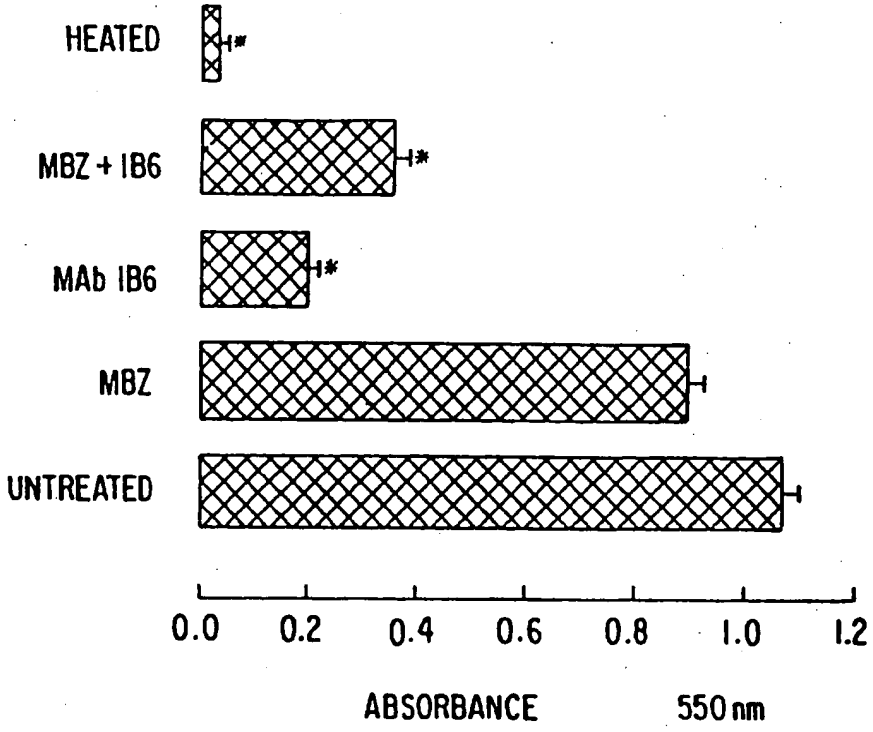


FIG. 3 A

FIG. 3 B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1.2
ABSORBANCE
550 nm

FIG. 4

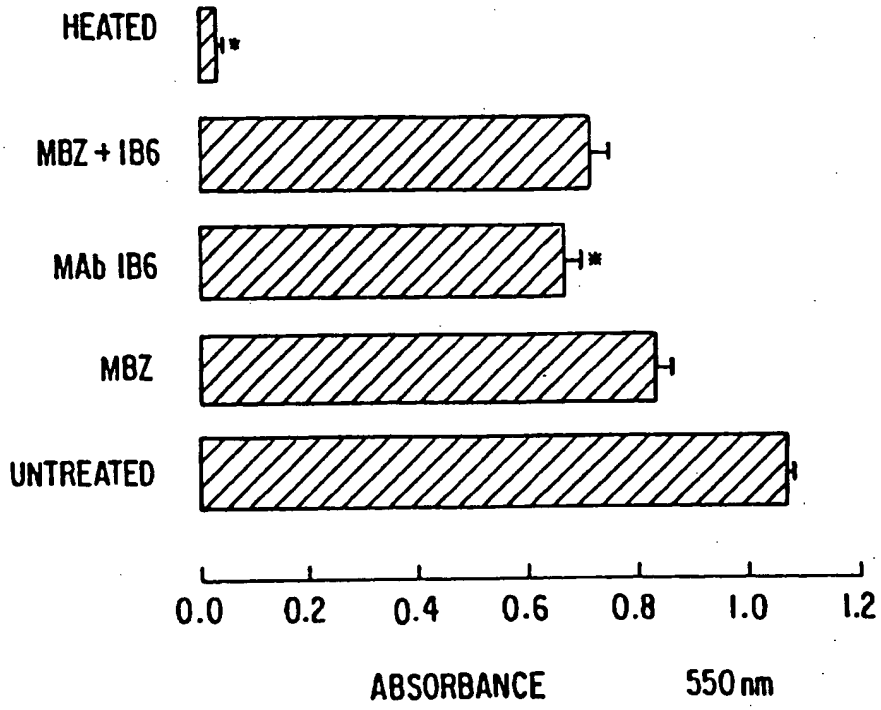
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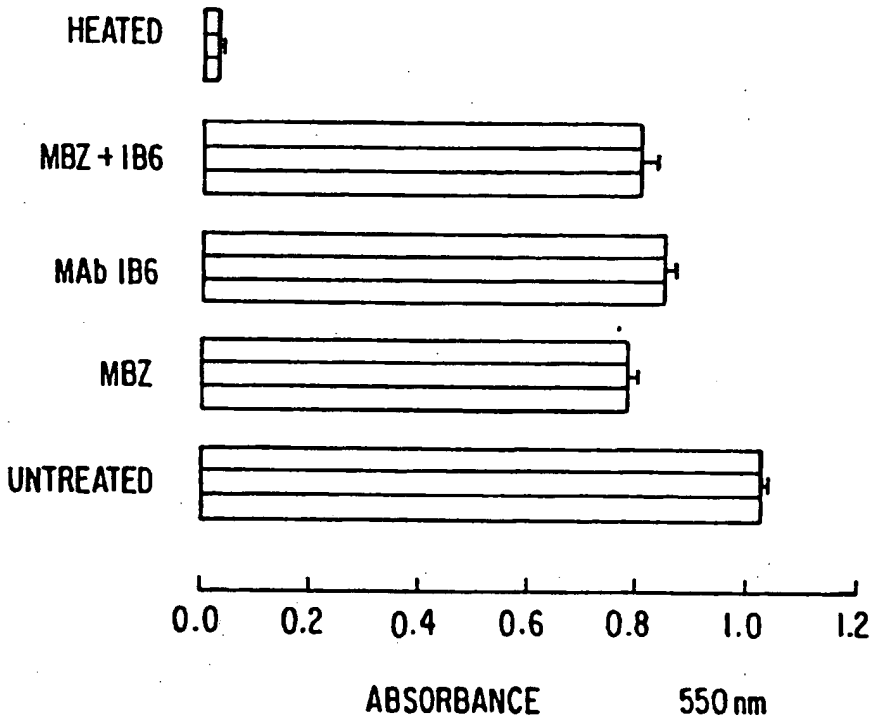
FIG. 5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ABSORBANCE
550 nm

FIG.6

FIGURE 7

NUCLEOTIDE SEQUENCE OF FILARIAL β-TUBULINS

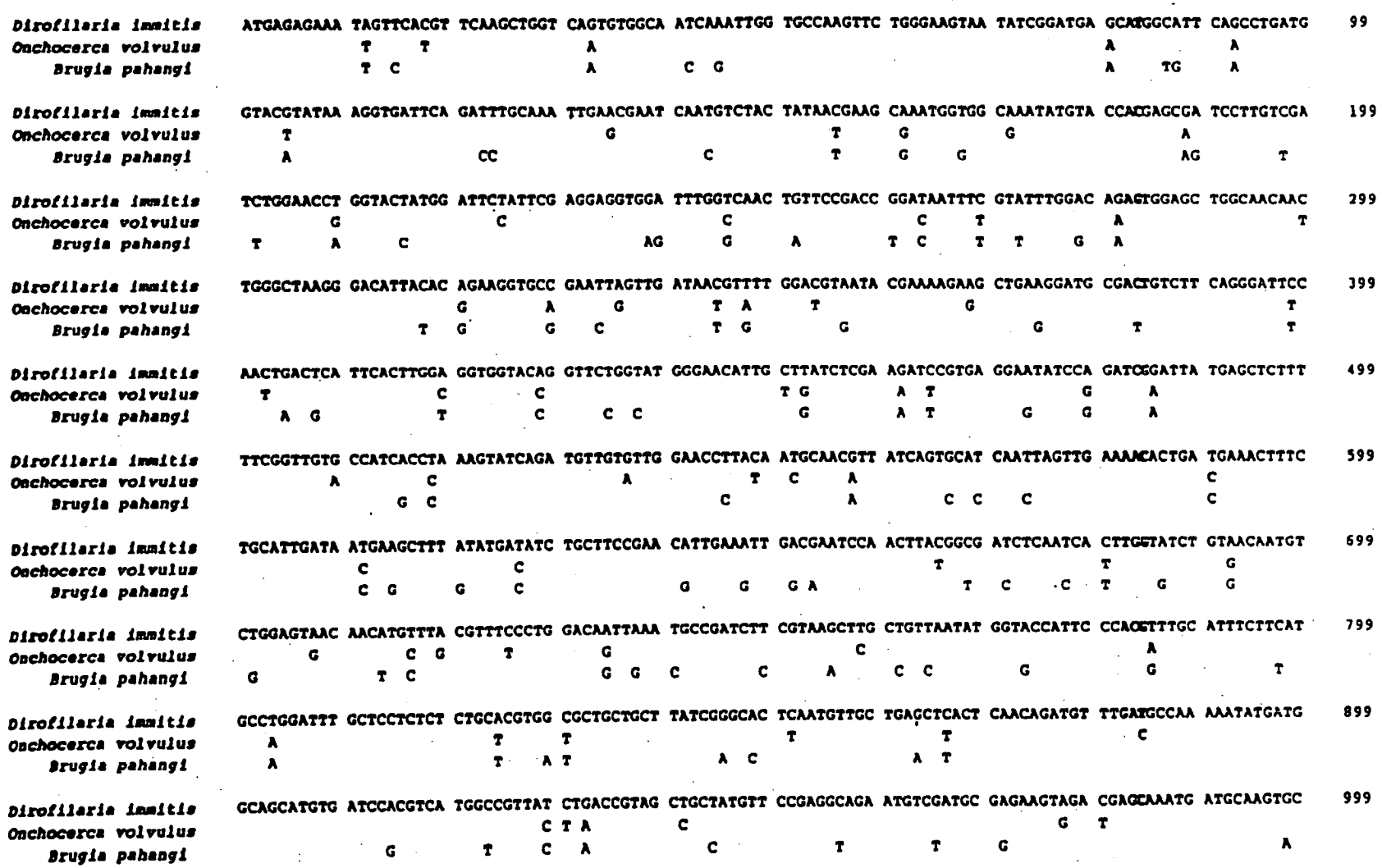

FIGURE 7 (Continued)
NUCLEOTIDE SEQUENCE OF FILARIAL β-TUBULINS

Dirofilaria immitis	AGATAAGAA TTCATCGTAT TTCGTTGAAT GGATTCCGAA TAACGTAAAA ACAGCTGTTT GCGATATTCC ACCACGTGGC TTGAAGATGA GCGCAACATT	109
Onchocerca volvulus	C T A A G C A A G	
Brugia pahangi	T A G C A A	
Dirofilaria immitis	CATCGGCAAT ACAACAGCCA TACAAGAACT TTTTAAACGC ATTTCTGAAC AGTTTACTGC TATGTTCCGA CGTAAAGCAT TCTTGCATTG GTATACTGGA	1199
Onchocerca volvulus	A C G C C C	
Brugia pahangi	T T A T C G C C C C	
Dirofilaria immitis	GAAGGTATGG ATGAAATGGA ATTCACGGAA GCAGAGAGTA ACATGAATGA CTTGGTGTCT GAATATCAGC AATATCAGGA TGCAACGTCT GATGAAGACG	1299
Onchocerca volvulus	T A T C A G A G T A	
Brugia pahangi	T G TG T C A G G A	
Dirofilaria immitis	GTGATCTTCA CGAAGGTGAA TCGGAATATA TTGAGCAAGA GGAATAA	1346
Onchocerca volvulus	C C A G A G	
Brugia pahangi	C A G A G G	

FIGURE 8

AMINO ACID SEQUENCE OF FILARIAL β-TUBULINS

Dirofilaria immitis	MREIVHVQAG QCGNQIGAKF WEVISDEHGI QPDGTYKGDS DLQIERINVY YNEANGGKYV PRAILVDLEP GTMDSIRGGG FGQLFRPDNF VFGQSGAGNN	99
Onchocerca volvulus	V E	
Brugia pahangi		
Dirofilaria immitis	WAKGHYTEGA ELVDNVLDVI RKEAEGCDCL QGFQLTHSLG GGTGSGMGTL LISKIREEYP DRIMSSFSVV PSPKVSDVVL EPYNATLSVH QLVENTDETF	199
Onchocerca volvulus		
Brugia pahangi		
Dirofilaria immitis	CIDNEALYDI CFRTLKLTNP TYGDLNHLVS VTMSGVTTCL RFPGQLNADL RKLAVNMVPF PRLHFFMPGF APLSARGAAA YRALSVAELT QQMFDAKNMM	299
Onchocerca volvulus	A D	
Brugia pahangi		
Dirofilaria immitis	AACDPRHGRY LTVAAMFRGR MSMREVDEQM MQVQNKNSSY FVEWIPNNVK TAVCDIPPRG LKMSATFIGN TTAIQELFKR ISEQFTAMFR RKAFLHWYTG	399
Onchocerca volvulus	I	
Brugia pahangi		
Dirofilaria immitis	EGMDEMEFTE AESNMNDLVS EYQQYQDATS DEDGDLQEGE SEYIEQEE	447
Onchocerca volvulus	A DEA	
Brugia pahangi	V A E	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04838

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 39/00
US CL : 424/265.1, 266.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/265.1, 266.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, Medline, Biosis, Derwent

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Guenette et al. Characterization of a b-tubulin gene and b-tubulin gene products of Brugia pahangi. Molecular and Biochemical Parasitology. 1991, Vol.44, pages 153-164, see entire document.	1-20
X --- Y	WO, 92/03549 A1(EURO-DIAGNOSTICS B.V.) 05 March 1992, see entire document.	1-10 --- 11-20
X	Helm et al. Localization and immunogenicity of tubulin in the filarial nematodes Brugia malayi and Brugia pahangi. Parasite immunology. 1989, Vol. 11, pages 479-502, see page 495, figure 7.	5, 7, 9, 12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
02 JULY 1996

Date of mailing of the international search report
24 JUL 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 308-4242

Authorized officer
KHALID MASOOD
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04838

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,842,999 A (FULLER et al) 27 June 1989, Column 3, 5, 11.	11-20
X	Geary et al. Three b-tubulin cDNAs from the parasitic nematode. Mol. Biochem. Parasitol. 1992, Vol. 50, pages 295-306, see entire document.	1-10
A	Platkin et al. Vaccinea. Philadelphia: W.B. Sanders Co. 1988, pages 568 to 575.	1-20